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The withdrawal of the objection to the specification because of alterations that were not initialed and/or dated, in view of applicants' response, is acknowledged.

The withdrawal of the rejection of claims 15-21 and 32-36 under the judicially created doctrine of obviousness type double patenting over claims 4, 7, and 9-11 of U.S. Patent No. 5,217,861, in view of the Terminal Disclaimer filed on May 12, 1994, is acknowledged.

The withdrawal of the rejection of claims 31 and 35 under 35 U.S.C. § 112, second paragraph, as being allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention, in view of applicants' amendments to the claims, is acknowledged.

The withdrawal of the rejection of claims 29-31 under 35 U.S.C. § 101 as claiming the same invention as that of claims 1-3 and 8 of prior U.S. Patent No. 5,217,861, in view of applicants' arguments, is acknowledged.

The present invention is directed to antibodies which bind with p12 or p18 protein of HIV-1, mixtures of antibodies which bind with p12, p15, p18, p25, p36, p42, or p80 protein of HIV-1, and immune complexes comprising p12 or p18 protein of HIV-1.

Claims 29-31 were rejected under 35 U.S.C. § 101 because the claimed invention allegedly lacks patentable utility. Office Action at 3. Applicants respectfully traverse this ground for rejection.

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Claims 29-31 are directed to immune complexes comprising p12 or p18 protein of HIV-1. In a Response filed April 26, 1994, applicants argued that an "immune complex" is a composition of matter that satisfies the statutory requirements for patentability, including utility under 35 U.S.C. § 101. As an example of the usefulness of the complexes, applicants noted that they can be used to obtain the component antigen or antibody. Galfre et al., "Preparation of Monoclonal Antibodies: Strategies and Procedures," Methods in Enzymology, 73, 3-46 (1981), was cited to show that techniques for producing and using immune complexes, and techniques for preparing and isolating antibodies and immune complexes, were known in the art.

Applicants also analogized the claimed immune complexes to a chemical intermediate useful for obtaining another compound, *i.e.*, the component antibody or antigen. In re Irani, 487 F.2d 924 (C.C.P.A. 1973), cited by applicants, held that the use of a chemical intermediate to produce another chemical compound satisfies the statutory requirement of utility for the chemical intermediate.

In response to applicants' arguments, the Examiner stated that the complexes *per se* do not have utility:

> There is no intended use of the complexes *per se*. . . .
> The reference cited by Applicant teaches using the antigens in immunoassays for detecting the presence of antibodies in a sample. This is in support of the Examiner's position that

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> the complexes are merely the result of an immunoreaction and have no utility of their own. . . . The analogy of an immune complex as a chemical intermediate is incorrect. In the intermediate of *Irani*, the compounds actually undergo a chemical change. In the case of immune complexes, the antigen and antibody are in close association but are otherwise unchanged. Therefore, the precedence of *Irani* is not pertinent. The claimed complexes have no patentable utility.

Office Action at 4. Applicants respectfully disagree.

Applicants maintain their position that the immune complexes are useful to obtain the component antigen or antibody. Applicants fail to understand why the U.S. Patent and Trademark Office has apparently made a determination that immune complexes *per se* lack utility, and therefore are unpatentable. The antigen and antibody are useful to detect the presence of antibody against the antigen or antigen against the antibody, respectively, in a biological sample. In contrast to these uses, immune complexes comprising antigen and antibody are useful to obtain the component antigen or antibody.

Applicants also disagree with the Examiner's characterization of In re Irani. It is acknowledged that the formation of an immune complex usually does not irreversibly alter the component antigen or antibody. This, of course, is the precise reason why the complexes are useful to obtain the component antigen or antibody: the reaction is reversible.

In re Irani did not hold that the chemical intermediate need be chemically altered to be patentable. In Irani, an acid

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was converted to an anhydride, which could then be converted back to the acid in a reversible reaction. Irani at 926. The court held that the anhydride was therefor useful to obtain the acid, which was useful as a sequestering agent and as a stabilizer. Irani at 926.

Furthermore, just as the anhydride and acid of Irani have different chemical and physical properties, the claimed immune complexes and the component antigen and antibody have different chemical and physical properties. For example, the interaction of antigen with antibodies can result in a variety of consequences, including precipitation (if the antigen is soluble), agglutination (if the antigen is particulate), and activation of complement. E. Benjamini and S. Leskowitz eds., Immunology: A Short Course, Second Edition, page 101 (Wiley-Liss, Inc., New York, 1991) (Exhibit 1). Cross-linking of various antigen molecules by antibody with the formation of an immune complex is required for precipitation, agglutination, or complement fixation. Immunology: A Short Course at 102. Cross-linking does not occur in the presence of antigen or antibody alone. The presence of complex is required to initiate cross-linking. This physical characteristic of immune complexes is important, for example, for the activation of complement. Immunology: A Short Course at 136.

Because applicants' immune complexes are a composition of matter having unique chemical and physical properties, useful for obtaining the component antigen and antibody, this subject

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matter satisfies the usefulness requirement of 35 U.S.C. § 101. Withdrawal of this ground for rejection is courteously requested.

The specification was objected to and claims 15-36 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to adequately teach how to make and/or use the invention, *i.e.*, failing to provide an enabling disclosure. Office Action at 4. Applicants respectfully traverse this ground for objection to the specification and rejection of the claims.

An Examiner may properly lodge a rejection of claims as based on a specification that is not in compliance with § 112, first paragraph, if it appears reasonable to conclude that one skilled in the art would have been unable to make or use the invention at the time the application was filed. When that conclusion is reasonable, the burden is on the applicant to rebut it, if he can, such as by offering evidence. In re Eynde, 178 U.S.P.Q. 470, 474 (C.C.P.A. 1975). The Examiner has not met this burden.

In the Office Action dated January 26, 1994, Paper No. 8, the Examiner stated that

> [t]he specification is not enabled for isolated and purified antibodies. There are no teachings in the specification for isolated and purified antibodies which react with p12, p15, p18, p25, p36, p42, and p80 proteins.

Paper No. 8 at 4-5. Applicants courteously disagree.

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As noted above, the claimed subject matter is directed to antibodies which bind with p12 or p18 protein of HIV-1, mixtures of antibodies which bind with p12, p15, p18, p25, p36, p42, or p80 protein of HIV-1, and immune complexes comprising p12 or 18 proteins of HIV-1. This subject matter is described in and enabled by the specification.

The detection and isolation of antibodies against p12, p18, p25, p36, p42, and p80 proteins of HIV-1 is described at page 13, lines 7-10 of the specification:

> [a]ntibodies to p12, p13, p18, p19, p25, p36, p42, and to p80 proteins have been detected in the sera of lymphadenopathy and AIDS patients . . .

Furthermore, the use of the disclosed proteins of HIV-1 as immunogens to produce antibodies against these proteins is also described in the specification at page 4, lines 1-5:

> [t]he proteins may be used . . . as immunogens for the production of antibodies for detection of proteins associated with the retrovirus . . .

See also page 26, lines 15-21 of the specification.

Furthermore, isolation of immune complexes comprising proteins of HIV-1 and antibodies against the proteins on a polyacrylamide electrophoresis gel is shown in Figure 5. Specification at page 3, lines 23-24; and page 22, line 23 through page 23, line 6; and Figure 5.

Similarly, isolation of immune complexes comprising p25 on a polyacrylamide gel is described at page 10, lines 11-27 and particularly, lines 21-25:

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> [i]mmunocomplexes were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. A p25 protein present in the virus-infected cells . . . was specifically recognized by serum from patient 1 . . .

Under analogous conditions, immune complexes comprising p12, p15, p18, p36, p42, and p80 were also isolated on polyacrylamide gels. Specification at page 11, lines 6-27.

Accordingly, antibodies against p12, p15, p18, p25, p36, p42, and p80 proteins of HIV-1, and immune complexes comprising p12 or 18 protein of HIV-1, are described in the specification.

Continuing, the Examiner stated that applicants' specification was not enabled for the production of monoclonal antibodies. Paper No. 8 at 4-5. Applicants courteously disagree.

As noted above, applicants teach that p12, p15, p18, p25, p36, p42, and p80 proteins of HIV-1 can be used to produce antibodies against these proteins. Specific techniques for the production of monoclonal antibodies were known in the art at the time the application was filed, and such techniques need not be described in the specification to satisfy 35 U.S.C. § 112, first paragraph.

The specification need not include that which is already known by and available to the public. Paperless Accounting, Inc. v. Bay Area Rapid Transit System, 804 F.2d 659, 664 (Fed. Cir. 1986). In fact, techniques that were old and well-known when the application was filed need not be included in the

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specification, and are preferably omitted. Spectra-Physics, Inc. v. Coherent, Inc., 827 F.2d 1524, 1534 (Fed. Cir. 1987).

The production of monoclonal antibodies is described in Galfre et al., "Preparation of Monoclonal Antibodies: Strategies and Procedures," Methods in Enzymology, vol. 73, 3-46 (Academic Press, Inc. 1981) (Exhibit 2). Materials for tissue culture are described at pages 4-8; immunization of animals is described at pages 8-10; the choice of myelomas is described at pages 10-13; experimental procedures are described at pages 14-32; the direct detection of antibody-secreting cells is described at pages 32-34; cloning is described at pages 34-37; and the selection of positive clones is described at pages 37-38. This subject matter is also described in Hurn et al., "Production of Reagent Antibodies," Methods in Enzymology, vol. 70, 104-142 (Academic Press, Inc. 1980) (Exhibit 3). Specifically, antibody production by hybridomas is described at pages 135-141.

Accordingly, because techniques for producing monoclonal antibodies were known in the art at the time the application was filed, such techniques need not be described in the specification. This is true for the enablement and written description requirements of 35 U.S.C. § 112, first paragraph. As stated by the court in In re Bosy, 149 U.S.P.Q. 789, 792 (C.C.P.A. 1966): "That which is common and well known is as if it were written out in the patent."

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Finally, the Examiner stated that the specification is not enabled for labeled antibodies. Office Action at 4-5. Applicants courteously disagree.

While applicants disagree with this ground for rejection, claims 17 and 21, directed to labeled antibodies, have been cancelled. In addition, claim 31, directed to a labeled immune complex, has been amended to recite labeling of the protein. These amendments have been made for the sole purpose of advancing the prosecution of the present application.

Because applicants' invention is described in and enabled by the specification, the withdrawal of this ground for objection to the specification and rejection of the claims is respectfully requested.

Claims 1-5, 7, and 8 were rejected under 35 U.S.C. § 102(a) as being allegedly anticipated by Barre-Sinoussi et al. Office Action at 5. Applicants respectfully traverse this ground for rejection.

Claims 1-5, 7, and 8 are not pending in this application. Withdrawal of this ground for rejection is respectfully requested.

Claims 15-21 and 32-36 were rejected under 35 U.S.C. § 103 as being allegedly unpatentable over Barre-Sinoussi et al. Office Action at 5. Applicants respectfully traverse this ground for rejection.

In the Amendment in Response to Paper No. 8, applicants noted that Barre-Sinoussi et al. was not available as prior art

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against the present application because applicants' claimed priority dates of GB 83 24800, filed September 15, 1983, and SA 84 7005, filed September 6, 1984, predate the publication of Barre-Sinoussi et al. in 1985.

In response, the Examiner stated that

> [t]his is not convincing as the priority documents are enabled only for p25. The South African and Great Britain patent applications do not provide teachings of the antigens taught in Barre-Sinoussi et al. Therefore, the priority documents do not remove the rejection from the instant invention.

Office Action at 5. Applicants disagree.

Applicants' priority documents, GB 83 24800 and SA 84 7005, describe p12, p15, p18, p25, p36, p42, and p80 proteins of HIV-1. For example, isolation of p12, p15, p18, p25, p42, and p80 proteins of HIV-1 on a polyacrylamide gel is described in GB 83 24800 at page 7, line 30 through page 7, line 31, and in SA 84 7005 at page 7, line 30 through page 8, line 32. Antibodies and against the HIV-1 proteins, and immune complexes comprising the proteins, are described at, for example, page 21, lines 32-39 of GB 83 24800, and at page 21, lines 32-39 of SA 84 7005.

Because applicants' priority documents provide support for the claimed invention, and because the priority documents were filed before the publication of Barre-Sinoussi et al., this reference is not available as prior art against this application. Withdrawal of this ground for rejection is courteously requested.

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Applicants acknowledge that this Amendment is submitted after final rejection. Because this Amendment places the application in condition for allowance, entry thereof by the Examiner is respectfully requested. Reconsideration and reexamination of this application and allowance of the pending claims at the Examiner's convenience are courteously requested.

The Commissioner is hereby authorized to charge any fees associated with this Amendment to our Deposit Account No. 06-0916. If a fee is required for an Extension of Time under 37 C.F.R. § 1.136 not accounted for above, such extension is requested and should also be charged to our Deposit Account.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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By: Michele M. Schafer
Michele M. Schafer
Reg. No. 34,717

Dated: October 11, 1994

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SECOND EDITION

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CHAPTER
7

ANTIGEN–ANTIBODY INTERACTIONS

INTRODUCTION

Antibodies constitute the humoral arm of acquired immunity that provides protection against infectious organisms and their toxic products. Therefore, the interaction between antigen and antibody is of paramount importance. Because of the exquisite specificity of the immune response, the interaction between antigen and antibody in vitro is widely used for diagnostic purposes, for the detection and identification of either antigen or antibody. The utilization of the in vitro reaction between antigen and serum antibodies is termed ***serology***. An example of the use of serology for the identification and classification of antigens is the ***serotyping*** of various microorganisms by the use of specific antisera.

The interaction of antigen with antibodies can result in a variety of consequences, including ***precipitation*** (if the antigen is soluble), ***agglutination*** (if the antigen is particulate), and ***activation of complement***. All of these outcomes are caused by the interactions between multivalent antigens and antibodies that have at least two combining sites per molecule.

The consequences of antigen-antibody interaction listed above do not represent the primary interaction between antibodies and a given epitope but, rather, depend on secondary phenomena, which result from the interactions between multivalent antigens and antibodies. Such phenomena as the formation

of precipitate, agglutination, and complement fixation would not occur if the antibody with two or more combining sites reacted with a hapten (i.e., a unideterminant, univalent antigen), nor would these secondary phenomena occur as a result of the interaction between a univalent fragment of antibody, such as Fab, and an antigen, even if the antigen is multivalent. The reasons for these differences are depicted in Figure 7.1A-E. ***Cross-linking*** of various antigen molecules by antibody is required for precipitation, agglutination, or complement fixation, and it is possible only if the antigen is multivalent and the antibody is divalent [either intact, or $F(ab')_2$] (see Fig. 7.1B,D,E). In contrast, no

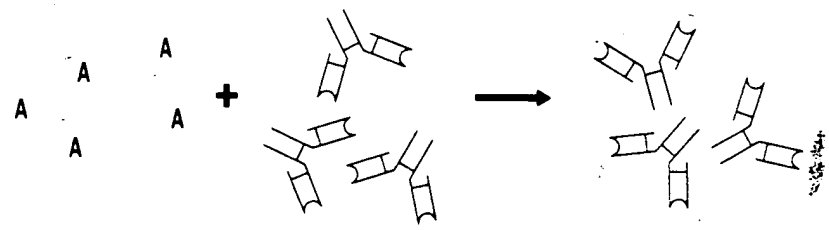

Figure 7.1A.
The reaction between antibody and a hapten.

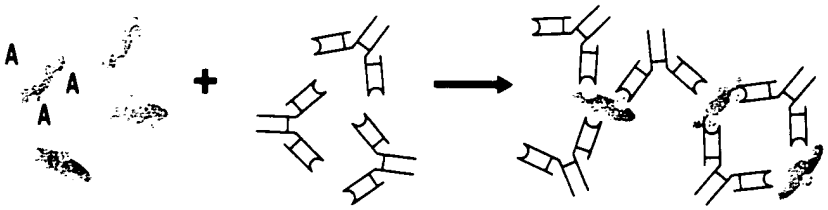

Figure 7.1B.
The reaction between antibody and a unideterminant, multivalent antigen.

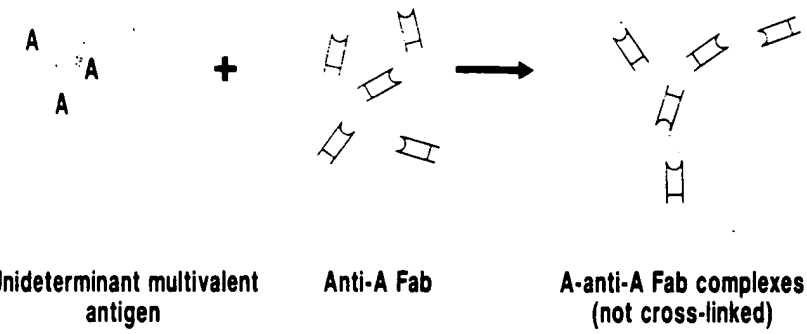

Figure 7.1C.
The reaction between Fab and a unideterminant, multivalent antigen.

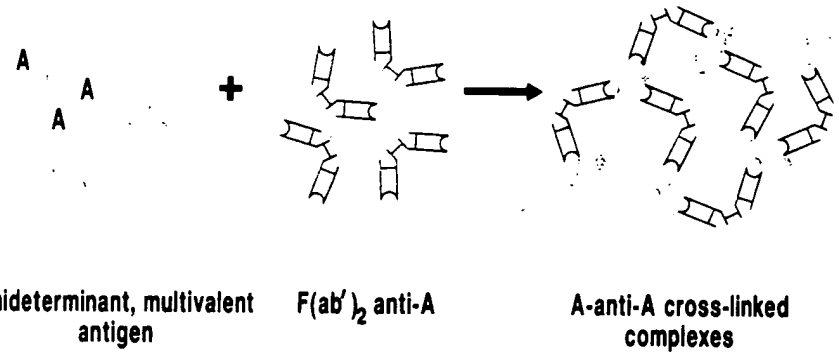

Figure 7.1D.
The reaction between $F(ab')_2$ and a unideterminant, multivalent antigen.

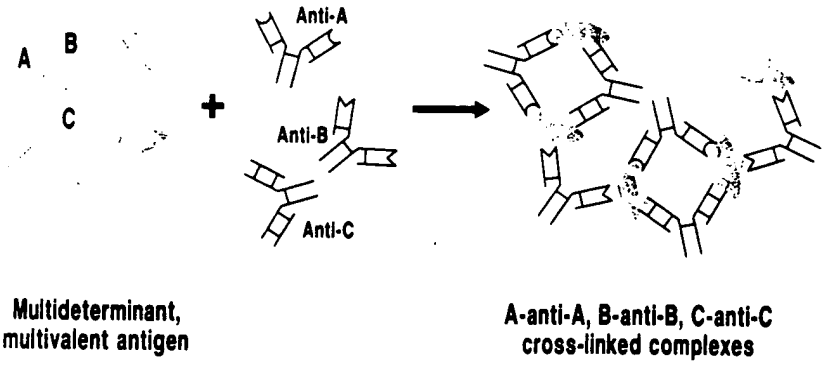

Figure 7.1E.
The reaction between antibodies to determinants A, B, and C, and a multivalent, multideterminant antigen with determinants A, B, and C.

cross-linking is possible if the antigen or the antibody is univalent (Fig. 7.1A,C).

There are many serological reactions that demonstrate the binding between antigen and antibodies. This chapter describes selected reactions that are used in diagnosis; many others, not included here, are mostly variations of the reactions described here.

PRIMARY INTERACTIONS BETWEEN ANTIBODY AND ANTIGEN

No covalent bonds are involved in the interaction between antibody and an epitope. Consequently the binding forces are relatively weak. They consist mainly of ***van der Waals forces, electrostatic forces,*** and ***hydrophobic forces,*** all of which require a very close proximity between the interacting moieties. Thus the interaction requires a very close fit between an epitope and the antibody, a fit that is often compared to that between a lock and key.

Because of the low levels of energy involved in the interaction between antigen and antibody, antigen-antibody complexes can be readily ***dissociated*** by ***low or high pH,*** by ***high salt concentrations,*** or by ***chaotropic ions,*** such as cyanates, which efficiently interfere with the hydrogen bonding of water molecules.

Association Constant

The reaction between an antibody and an epitope of an antigen is exemplified by the reaction between antibody and a univalent hapten. Because an antibody molecule is symmetrical, with two identical Fab antigen combining sites, one antibody molecule binds with two identical hapten molecules, each Fab binding in an independent fashion with one hapten molecule. The binding of a hapten (H) with each site can be represented by the equation

$$Ab + H \rightleftharpoons AbH$$

and the association constant between the reactants is expressed as

$$K = \frac{[AbH]}{[Ab][H]}$$

serum do not increase after immunization. Like antibodies, they appear to have arisen late in evolution and are found only in vertebrates.

Bordet discovered that the action of complement, in the presence of the appropriate antiserum, results in the lysis of red blood cells. Based on this observation, Bordet developed the ***complement fixation test.***

We now know that in addition to lysis, the activation of complement results in the generation of many powerful biologically active substances.

This chapter describes the complement system, the properties of the complement components and their biological relevance, and the complement fixation test.

THE COMPLEMENT SYSTEM

Nature has devised two pathways for the activation of complement, the so-called ***classical pathway*** and the ***alternative pathway***. Although both pathways share some common components, they differ in the ways in which they are initiated. The classical pathway requires antigen-antibody complexes for initiation, while the alternative pathway does not. In the sections that follow, both pathways, their activation, and their products will be described.

The Classical Complement Pathway

The ***classical complement system*** involves the activation, in an orderly fashion, of nine major protein components designated as C1 through C9. For several steps in the activation process, the product is an enzyme that catalyzes the subsequent step. This cascade provides amplification and activation of large amounts of complement by a relatively small initial signal. To safeguard against the system going awry, the series of reactions also provides several sites for regulation.

The sequence of the classical complement pathway is given in Figure 8.1 and is discussed below.

ACTIVATION OF C1. The first component to become activated is the C1 complex, consisting of three proteins, C1q, C1r, and C1s, with molecular weights of approximately 400,000, 95,000, and 85,000 daltons, respectively. The complex is held together by calcium ions.

[1] Preparation of Monoclonal Antibodies: Strategies and Procedures

By G. GALFRÈ and C. MILSTEIN

I. Introduction

The derivation of cell lines capable of permanent production of specific antibody directed against a predefined immunogen was first reported in 1975.[1] The method was based on fusion between myeloma cells and spleen cells from suitably immunized animals. Spleen cells die in a short time under ordinary tissue culture conditions. Myeloma cells have been adapted to grow permanently in culture, and mutants were isolated that lacked the enzymes hypoxanthine guanine ribosyltransferase (azaguanine-resistant) or thymidine kinase (bromodeoxyuridine-resistant). Such mutants cannot grow in medium containing aminopterin and supplemented with hypoxanthine and thymidine (HAT medium) because they are unable to utilize the salvage pathway. Hybrids between such cells and spleen cells can be selected from the parental components as the only cells that actively multiply in HAT selective medium. From the growing hybrids, individual clones can be selected that secrete the desired antibodies. Such antibodies are therefore of monoclonal origin. The selected clones, like ordinary myeloma lines, can be maintained indefinitely.

This basic methodology has been used to prepare antibodies against a large variety of antigens. These include antibodies to haptens, small natural products with biological activity, such as neuropeptide and peptide hormones, enzymes and other proteins, polysaccharides, glycoproteins, lipopolysaccharides, histocompatibility antigens, differentiation antigens and other cell surface antigens, viruses, etc. The results justify the idea that the production of any antibody synthesized by the immunized animal can be immortalized by cell fusion methods.

Cell fusion is therefore a way of immortalizing cells expressing a transient differentiated function. The outcome of the fusion between a given cell line and a heterogeneous population of normal cells is affected by the phenotype of the particular cell line used. Fusions with myelomas result in a high frequency of antibody-secreting hybrids. On the other hand, other cell lines, for example, T cell lymphomas, are used for the immortalization of other differentiated properties, such as T cell functions. The derivation of such hybrids is based on the same general principles. Since the out-

[1] G. Köhler and C. Milstein, *Nature* (*London*) **256**, 495 (1975).

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come is not an antibody that can be used as a general reagent, it will not be discussed further.

The derivation of permanent lines of hybrid cells producing monoclonal antibodies (McAb) exhibiting certain desired properties presents widely different degrees of difficulty. Desired properties include not only specific recognition of an antigen: other no less critical properties are the fine specificity of the antibody, avidity and kinetic parameters important for radioimmunoassays, cytotoxic properties necessary for direct complement-dependent lysis, etc. When an animal is injected with a given antigen its usual response is the production of a highly heterogeneous population of antibodies directed against the immunogen. Among these many antibodies, some may have the desired properties but will be mixed with many others that will express alternative or undesirable properties. When McAb are prepared by the hybrid myeloma method, the collection of clones randomly derived represents a cross section of such a heterogeneous population. In addition the overall response of the individual animal can be strong or very weak, and this will be reflected in the proportion of hybrid clones producing the desired antibody within the total population of actively growing hybrids. These considerations are paramount in the preliminary estimation of the degree of difficulty that may be involved in the derivation of specific reagents. No less important is the fact that the characteristics of the McAb that will be derived will depend to a large extent on the way the whole experiment was originally designed. In this chapter we will attempt to provide guidelines for the derivation of specific McAb. For this we will draw on our own experience and will only occasionally refer to protocols and approaches that are not in use in our own laboratory. This is not because we consider our experience more valid than that of others, but, as in many complex operations, the final blend reflects the unique experience of the particular laboratory.

II. Materials for Tissue Culture[2]

A. Media

Tissue culture grade water is used throughout. This is usually deionized and double-distilled over glass.

For the preparation of McAb the most commonly used media are Dulbecco's Modified Eagle's Medium (DMM) and RPMI-1640. For practical reasons it is better to standardize with one medium. Alternative media are often required for cell lines from other laboratories. Whichever medium is chosen, particular attention must be devoted to its preparation. We will discuss here only the use of DMM, but the same general principles apply to any media. DMM is commercially available in different forms.

1 × DMM: The ready-to-use DMM (1 × DMM) can be bought as complete medium to which only pyruvate or glutamine and extra components are added before use, following the manufacturer's instructions (1 × DMM, Gibco Europe, Glasgow, Scotland, Cat. No. 196G; or Flow Laboratories, Irvine, Scotland, Cat. No. 12-334-54). Different batches may vary slightly. It is therefore advisable to buy batches in sufficient quantities to allow work for a reasonable period. The 1 × media will in general decay during storage; they are kept in the dark at 4°. For critical operations it is advisable to use media that are not more than 3 months old—especially when, as is the case with the 1 × medium, the exact date of preparation is not known.

10 × DMM: Ten times concentrate solutions (10 × DMM, Gibco Europe, Glasgow, Cat. No. 330-2501; or Flow Laboratories, Irvine, Cat. No. 14-330-49) are also available. About 4 liters of tissue culture grade water are autoclaved in a 6-liter glass flask and allowed to cool to room temperature. The 10 × medium and other components as specified by the manufacturer are then added. If necessary the volume is adjusted to approximately 5 liters with water.

Powder DMM: Prepared from dry powder (Gibco Laboratories, Grand Island, New York, Cat. No. 430-2100) following the manufacturer's instructions. This requires filter sterilizing units of 20-liter capacity or larger.

We find media prepared directly from powder to be the best, probably because they are usually used when fresher. The 1 × medium is almost as good but is much more expensive and requires more 4° storage space. We favor a supply of a few liters of 1 × DMM for comparison and for emergency cases. The medium prepared from the 10 × concentrate is generally not as good, and the batches are more variable. We use it only on well established lines when our production capacity from powder medium cannot cope with large-scale cultures. Concentrated medium is necessary for cloning in soft agar or agarose, and it is best to prepare 2 × medium from dry powder.

HAT medium

100 × HT: 136.1 mg of hypoxanthine (Sigma, Poole, Dorset, England, Cat. No. H9377) and 38.75 mg of thymidine (Sigma, Cat.

[2] A more detailed discussion on procedures for tissue culture can be found in J. Paul, "Cell and Tissue Culture," 5th ed., Churchill-Livingstone, Edinburgh and London, 1975; and W. B. Jakoby and I. H. Pastan, this series, Vol. 58.

No. T9250) are suspended in about 50 ml of water, and 0.1 *M* NaOH is added dropwise until dissolved. Adjust volume to 100 ml. Store at −20°. Thaw at 70° for 10–15 min.

50 × HT: Dilute 100 × HT with 1 volume of DMM. Filter sterilize and store in 25 ml aliquots at 4°.

1000 × aminopterin: Aminopterin (Sigma, Cat. No. A2255) 17.6 mg/100 ml. Proceed as for 100 × HT.

50 × HAT: 50 ml of 100 × HT, 5 ml of 1000 × aminopterin, and 45 ml of DMM. Filter, sterilize, and store in 25-ml aliquots at 4°.

1 × HT and 1 × HAT [20% fetal calf serum (FCS)]: 500 ml of DMM, 100 ml of FCS, 12 ml of 50 × HAT or 50 × HT, and antibiotics as required

B. Additives for Contamination Control

The most common tissue culture contaminants are bacteria, yeast, and fungi. To control them there is no substitute for a good, sterile technique. Bacterial contamination is not generally difficult to control with appropriate antibiotics. However, the routine inclusion of antibiotics in the medium leads to the selection of resistant bacteria. Sometimes these are slow growing and difficult to detect and become permanent and undesirable guests in the laboratory. A good compromise is to have penicillin and streptomycin (Gibco Europe, penicillin–streptomycin, 5000 units/ml, Cat. No. 507, used at a final dilution of 50 units/ml) routinely included in the medium. Gentamycin (Flow Laboratories, Cat. No. 16-762-45) is then reserved to control outbreaks of penicillin–streptomycin-resistant bacteria in important experiments. Gentamycin is said to be effective for mycoplasma infections. We have not found such infections a common problem. We have not ourselves found a satisfactory control for yeast and fungi. Contamination with yeast usually occurs in isolated cultures and does not spread. Fungal contamination is more difficult to confine. Spores quickly spread in the plates, out into the incubators, and eventually into the whole room. Particular attention must therefore be devoted to separating and eliminating the infected cultures as soon as possible. It is definitely worthwhile to prepare duplicates of important cultures in separate plates as soon as feasible.

C. Choice of Serum

Special care in the choice of serum is essential. Sera from different sources vary greatly, and each batch must be properly tested. Because of its low immunoglobulin content, FCS does not generally interfere with the assay of specific McAb. This is the most important reason for using FCS,

but not the only one; FCS seems also to give the highest efficiency in the preparation of hybrids. Heat inactivation is not usually necessary but may be required in specific cases. Because of the high price and extreme shortage of FCS, alternatives are being sought. Most parental myelomas were originally adapted to grow in medium supplemented with heat-inactivated horse serum, and early fusions were prepared with it. This was found to give unacceptable backgrounds when screening for certain antibody activities. Horse serum devoid of its γ-globulin fraction has been suggested as one alternative, but a wider search is required.

For the fusion, selection, and cloning steps, we recommend medium containing 20% FCS. As soon as a hybrid is selected we routinely shift from 20% to 10% FCS in the medium. When cells are well adapted we take them to 5% FCS. At concentrations lower than 5% FCS, cells grow more slowly, and this can be advantageous for routine maintenance.

If heat-inactivation is required it should be done carefully. Frozen bottles are thawed quickly in a 37° bath and left at 37° to warm up. They are then transferred to a 56° bath and left for 30–45 min, depending on the size of the container, with occasional mixing.

Testing of Serum Batches. Careful testing of the quality of serum batches is recommended in all cases. This is easily done by growth-efficiency tests. We routinely use a limiting dilution method as follows: From a logarithmic growing culture of any cell line, preferably a hybrid not yet growing vigorously, prepare four tubes containing 2000, 1000, 500, and 250 cells/ml. Dispense 150 μl of medium containing 20% FCS that is to be tested into the wells of rows 1–6 of a 96-well microtiter plate (Sterilin, Teddington, Middlesex, England, flat-bottom microtiter plates, Cat. No. M29ARTL). In rows 7–12 apply an equal volume of medium containing a control FCS for comparison. A multidispenser (e.g., Hamilton, Cat. No. PB600) fitted with a 10-ml plastic syringe is convenient. Apply 20 μl of each cell suspension into 24 consecutive wells. This is conveniently done with a multidispenser fitted with disposable 1-ml syringes. (Plastic syringes must be trimmed at the ring head to fit the dispenser or, better, the dispenser syringe-holder must be cut to allow the plastic syringe to snap in.) The plate is wrapped in cling film (e.g., Alcan Wrap) to reduce the risk of contamination and is incubated at 37° in a CO_2 humid incubator. After 3 days wells are examined for the presence of live cells, and after 7–10 days for active growth.

D. Equipment

The essential requirements are common to ordinary tissue culture laboratories and include 37° incubators with and without a controlled atmosphere of CO_2 and humidity. The CO_2 concentration should be adjusted to

give a steady pH of 7.2 to a sample of medium in an open container. Sterile work benches, inverted and ordinary microscopes preferably with phase contrast, water baths and/or hot blocks thermostatically controlled (e.g., Tecam Dry Block 08-3), centrifuge, liquid N_2 storage, plastic and glassware. Other items of equipment range from highly desirable to luxurious and are listed when recommended.

For long-term continuous culture and for mass culture of cells we strongly favor spinner vessels. These are enclosed glass vessels of 1–20-liter capacity with ports for delivery and removal of liquids and air and a Teflon-coated magnetic bar clear of the bottom of the vessel. A convenient arrangement for long-term cultures is shown in Fig. 1, in which a water-jacketed unit is used. These units are better than the non-water-jacketed type in terms of reliability of temperature control, but they are more cumbersome and therefore less convenient for short-term mass cultures. Components should be glass or Teflon as far as possible. Flexible tubing must be tissue culture grade (e.g., silicon rubber). When metal parts cannot be avoided, these must be of stainless steel 18/8 grade.

Mouth-pipetting is not recommended. We use a pipette-aid (Drummond Scientific Co., supplied by Bellco Glass Inc., Cat. No. 1225-80122) to which a flexible rubber tubing is attached. In this way long, as well as short, pipettes can comfortably be used.

III. Parental Cells

The choice and preparation of the two types of cells that are used as parents during fusion is of paramount importance. In particular the immune state of the animal from which the spleen is taken can make all the difference between success and failure. For the rest of this chapter we will discuss only procedures utilizing spleen cells. However, other lymphoid organs can be used, particularly lymph nodes. Indeed in specific cases this may be a better alternative if used in conjunction with certain immunization protocols.[3]

A. Immunization of Animals

The purity of the immunogen per se is irrelevant. It becomes important only if (*a*) impure material gives weaker specific responses; (*b*) the methods of assay do not distinguish between antibodies to the specific component and antibodies to the impurities. Some antigens are immunodominant

[3] D. Zagury, L. Phalente, J. Bernard, E. Hollande, and G. Buttin, *Eur. J. Immunol.* 9, 1 (1979).

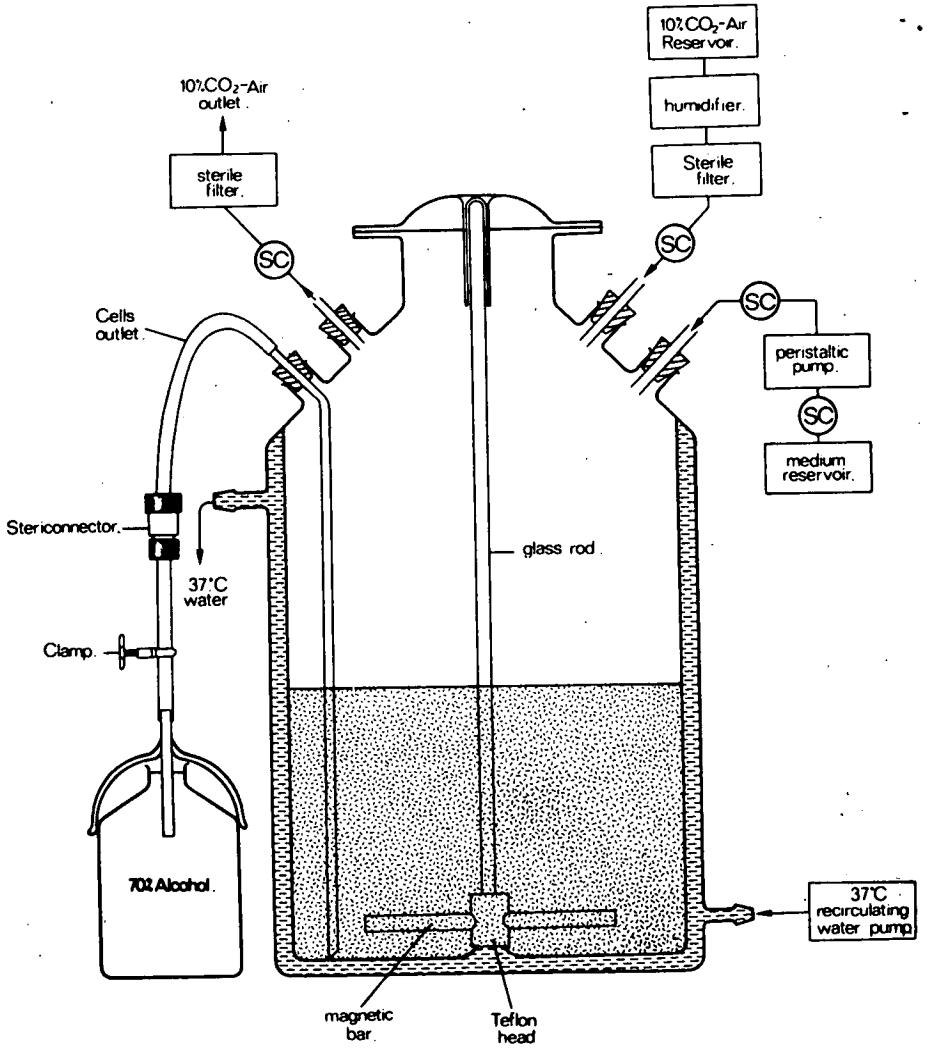

FIG. 1. Water-jacketed spinner vessel for continuous growth of cell lines. Simpler versions, with no water jacket, are used for short-term cultures and for 20-liter capacity (Cambridge Glassblowing, Crane Industrial Estate, or Wingent Engineering, Ltd., Cambridge, U.K.). SC are stericonnectors, size S 1/4 L.H. Engineering Co., Ltd., Stoke Poges, U.K.

and give strong immune responses even when present in only trace amounts. But the responsiveness of individual animals to the various chemical components of a mixture is rather variable, involving suppression as well as induction. There are so many factors to be taken into

consideration that exhaustive studies on the best immunization protocols are justified only in special cases. On the other hand, it is highly recommended that more than one immunization schedule should be tried, using several animals. Tests on different species and strains is a desirable practice. When no other information is available, note that the immunization protocol described below has been successful in many cases. In the final choice consideration should also be given to the species and strain of the parental myeloma. Interspecies hybrid lines are not suitable for production of antibodies in animals. If the animal chosen is of a strain different from that of the myeloma parent, the growth of tumor will require the use of F_1 hybrid animals. Other considerations being equal, the simplest animals to use are BALB/c mice and LOU rats.

Protocol. If the antigen is a soluble protein, a solution of about 1–5 mg/ml in saline is emulsified with an equal volume of Freund's complete adjuvant. This can be done by repeatedly squirting the suspension through the nozzle of a syringe. A total of about 0.3 or 0.6 ml is injected into multiple sites in mice and rats, respectively. The injections could be subcutaneous in at least three or four sites—for instance, in the back near the legs and the base of the tail. The treatment can be repeated at intervals of 3–5 weeks. About 10 days after each injection a drop of blood is taken by cutting the end of the tail of each animal, and this blood is used to test for the presence of specific antibodies. The animals giving the best antiserum are selected for fusion. After a rest period of a month or longer 0.2–0.4 ml of the protein solution (without Freund's adjuvant) is injected intravenously. The animals are sacrificed 3–4 days later, and the spleen cells are used as described in the fusion protocol.

The procedure can be speeded up by attempting a "blind" fusion using a primary immunized animal. In this case only an intravenous injection is performed 3–4 days before fusion.

B. Choice of Myelomas

The first point to consider is the species. Unless there are specific reasons against it, the myeloma should be of the same species as the immunized animal. This will permit easy development of tumors when hybrid myelomas have been derived. The choice between the rat and the mouse systems should be based on several considerations. The most important is the relative immune response to the antigen in question. If, after immunization of different rats and mice, individual animals show a better response, the myeloma parental cells should match the animal. If the responses are only marginally different, other considerations become important.

The rat system is better for the preparation of large amounts of antibody. Rats are considerably bigger than mice and just as easy to handle. Derivation of the hybrids with the rat lines has been found to be less straightforward than with the mouse lines, but with more experience the problems seem to disappear. On the other hand, the final recovery of positive clones from early hybrid cultures appears to be easier with rat lines. This may be because the percentage of growing hybrids expressing spleen immunoglobulins is 60% when mouse myeloma parental lines are used and over 90% with rat.[4] This is taken into consideration in the estimate of overall performance in Table I.

The next consideration is the chain composition of the myeloma. Hybrid myelomas codominantly express the immunoglobulin chains of both parental cells. If the myeloma line expresses both heavy and light chains of an immunoglobulin, the hybrid will express four chains. For convenience these are designated as follows: G and K are the heavy and light chains contributed by the parental myeloma; H and L are the respective chains (regardless of class or type) contributed by the spleen parental cells. Coexpression of chains from both parents *within* a single cell leads to the secretion of mixed molecular species. Thus, in addition to the parental types LHHL and KGGK, the hybrids will express immunoglobulin molecules of the type LHHK, KGGL, LGGL, and KHHK, regardless of class and type of chain. Moreover, mixed molecules containing both parental heavy chains of the type LHGK, KHGL, and all other permutations may also arise, but this depends on the class of the heavy chains. Although thorough investigations for all classes have not been carried out, the general rule seems to be that heavy chains of different subclasses, but not of different classes, can associate to form mixed molecules. For instance, $\gamma 1$ can combine with $\gamma 2a$ and $\gamma 2b$ but not with μ chains.

Hybrid myelomas of the type HLGK (i.e., expressing all four immunoglobulin chains) give rise with high frequency to mutant clones that no longer express one of the chains. This is not a random event, and the pattern of losses is shown in the diagram of Fig. 2. In Section IX, we describe the method for the derivation of segregants. It is much simpler to start with a myeloma that expresses only light chains. Such myelomas give rise directly to HLK hybrids (see diagram, Fig. 2). From here variants of the HL or HK type can be derived, but, particularly with the rat Y3 line, the frequency with which they arise is not so high. Using lines not expressing any myeloma chain (nonproducers), the hybrids will express only the antibody of the parental spleen. In Table I we give a subjective

[4] C. Milstein, M. R. Clark, G. Galfre, and A. C. Cuello, *in* "Immunology" (M. Fougereau, ed.), p. 17. Academic Press, New York, 1980.

TABLE I
PARENTAL MYELOMA LINES IN USE IN OUR LABORATORY

Name	Strain	Derived from	Immunoglobulin expression	Expected expression in hybrids	Estimated overall performance
Mouse lines					
P3-X63/Ag 8[a]	BALB/c	P3K[b]	MOPC 21[c] IgG_1 (κ)	HLGK[d]	++
NSI/1.Ag 4.1[e]	BALB/c	P3K	κ chains (nonsecreted)	HLK	++
X63/Ag 8.653[f]	BALB/c	X63/Ag 8	None	HL	++
Sp2/O[g]	BALB/c	Hybrid Sp2[h]	None	HL	+
NSO/1[i]	BALB/c	NSI/1.Ag 4.1	None	HL	+++
Rat lines					
Y3-Ag 1.2.3[j]	Lou[k]	R210.RCY3[l]	S210 κ chain[m]	HLK	+++
YB2/3.0 Ag 20[n]	(Lou × AO)F_1	Hybrid YB2/3[o]	None	HL	+++

[a] Köhler and Milstein.[1]
[b] K. Horibata and A. W. Harris, *Exp. Cell Res.* 60, 61 (1970).
[c] M. Potter, *Physiol. Rev.* 52, 631 (1972).
[d] See also Fig. 2.
[e] Köhler *et al.*[38]
[f] J. F. Kearney, A. Radbruch, B. Liesegang, and K. Rajewsky, *J. Immunol.* 123, 1548 (1979).
[g] M. Shulman, C. D. Wilde, and G. Köhler, *Nature (London)* 276, 269 (1978).
[h] Sp2 is a hybrid myeloma prepared with X63/Ag 8 and a spleen from a BALB/c mouse immunized with sheep red cells.[1]
[i] This is a subline of NSI/1.Ag 4.1 that does not express the intracellular light chains (M. Clark, B. W. Wright and C. Milstein, unpublished data, 1980).
[j] Galfre *et al.*[6]
[k] Obtainable from H. Bazin, Ph.D., Experimental Immunology Unit, Bte UCL 3056, Clos Chapelle-Aux-Champs 30, 1200 Brussels, Belgium.
[l] R. G. H. Cotton and C. Milstein, *Nature (London)* 244, 42 (1973).
[m] P. Queninjean, H. Bazin, A. Beckers, C. Deckers, J. F. Heremans, and C. Milstein, *Eur. J. Biochem.* 31, 354 (1972).
[n] B. W. Wright and C. Milstein, unpublished data, 1980.
[o] YB2/3 is a hybrid myeloma prepared with Y3 cells and a spleen from an A0 rat immunized with human complement (see Lachman *et al.*[22]).

FIG. 2. Chain composition of hybrids and the derivation of segregants. H and L are the heavy and light chains contributed by the cells from the immune animal, and G and K are the myeloma heavy and light chains. $\bar{0}$ refers to no intracellular or secreted chains. The plus sign (+) indicates specific antibody. Dashed lines indicate uncommon or never observed.

assessment of the "performance" of each line. It is an attempt to estimate the relative chances of success in the derivation of specific clones taking a number of factors into consideration.

It follows that as a general rule the nonproducer myelomas are the best choice, especially as lines with good performance are now available. However, there are cases where the artificial combinations may be a desirable by-product. For instance, an HLK hybrid from which the variant clones HL and HK are prepared could provide an antibody (HL) and its ideal negative control (HK). For the preparation of standard reagents for commercial distribution, this may prove to be highly desirable. Other examples where such hybrids may be useful are in the preparation and testing of anti-idiotypic antibodies and when mixed molecules can be used for specific purposes.

Maintenance of Myeloma Cells. Whatever is the choice of myeloma, the most important factor for the successful derivation of hybrids is the way in which the myeloma culture has been maintained prior to fusion. The goal is logarithmic growth for as long as possible, certainly not less than a week before fusion. We strongly advise the use of spinner cultures as opposed to stationary suspension cultures. This may be an essential requirement when using the rat line Y3, which tends to stick to the walls of the culture vessel. Some workers advise the use of trypsin or other enzymes to detach the cells, but we have no experience of this procedure.

IV. Experimental Procedures

A scheme of the general procedures involved in the derivation of monoclonal antibodies is presented in Fig. 3. A number of well defined separate steps can be identified. These will be discussed individually under separate headings. However, it must be emphasized that this is by no means a rigid general protocol. Variations can be introduced at almost every step. Some variations, however, may affect more than a single step, and this should be carefully considered at the experimental design stage.

A. Preparation of Parental Cells for Fusion

1. Spleen Cells

Materials

FCS-DMM, 2.5%: 500 ml of DMM, 12 ml of FCS
CO_2 chamber: a 2-liter beaker containing Dry Ice covered with paper towels, with an aluminum foil lid
Alcohol, 70%: Prepare about 300 ml in a 500-ml beaker
Round-bottom plastic tubes (e.g., Sterilin 142AS), 10 ml
Pestle from a round-tip Teflon homogenizer to fit very loosely (1 mm clearance) the round-bottom plastic tubes
Sterile dissection instruments (forceps, scissors)

Procedure

1. Kill the animal by placing it in the CO_2 chamber for 1–2 min.
2. Dip it in 70% alcohol. Place it on a board in a sterile cabinet, and remove the spleen under sterile conditions.
3. Put the spleen in a petri dish containing about 5 ml of 2.5% FCS-DMM kept on ice, and wash gently.
4. Transfer the spleen to a 10-ml round-bottom tube, cutting it into three or four pieces. Add 5 ml of fresh 2.5% FCS-DMM.
5. With the Teflon pestle squash the pieces gently to make a cell suspension.
6. Allow the remaining clumps and pieces of connective tissue to sediment for about 3 min, then transfer the cell suspension to a 10-ml round-bottom plastic tube.
7. Fill the tube with 2.5% FCS-DMM and spin at room temperature for 7–10 min at 400 *g*. (During this interval start the preparation of myeloma cells as below.)
8. Resuspend pellet in about 10 ml of fresh medium and centrifuge as above.

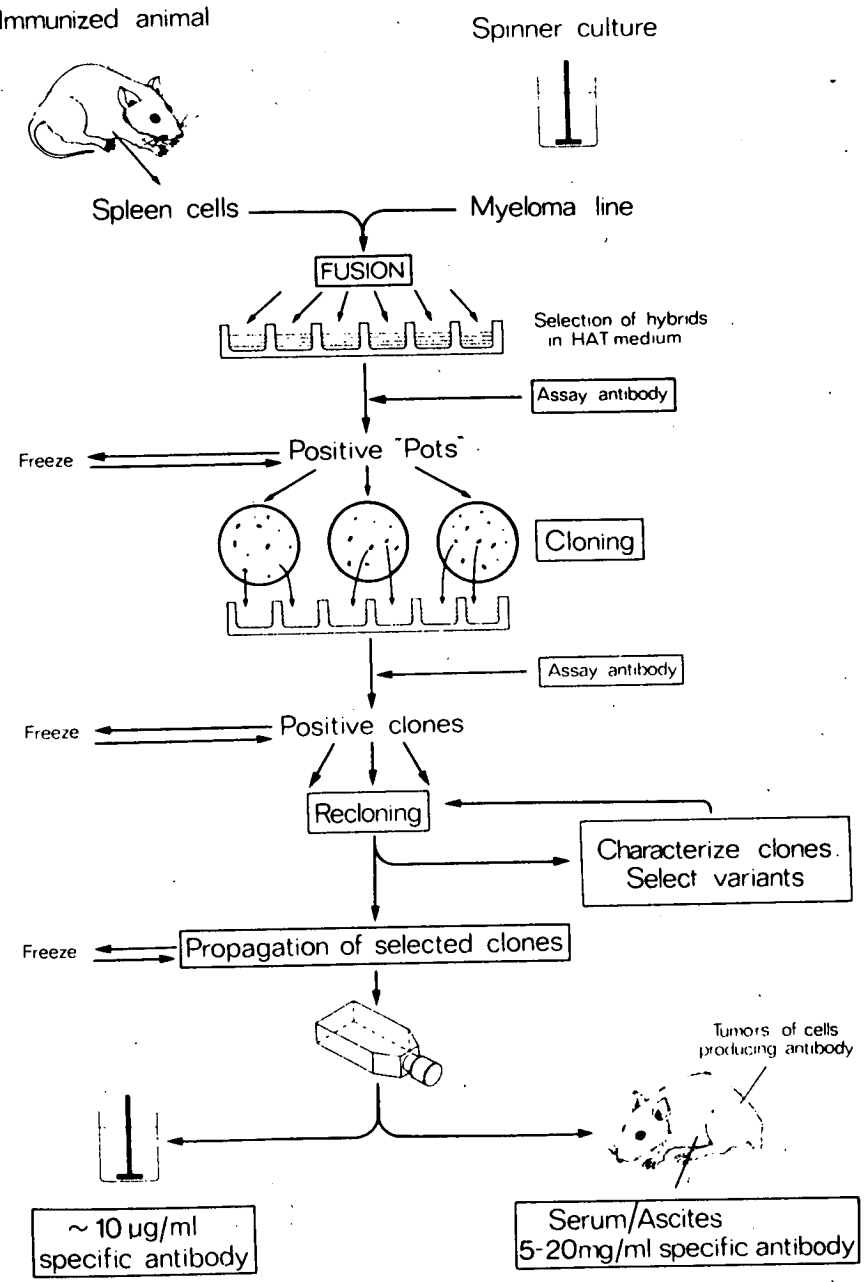

FIG. 3. Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas.

9. Resuspend pellet in 10 ml of medium, and count the cells. This suspension can also be used as a feeder layer for the culture of the fused cells.

Viability, determined by phase-contrast microscopic examination (or Trypan blue exclusion test), should be higher than 80%.

2. *Myeloma Cells*

Enough (see below) myeloma cells from a culture in logarithmic growth are pelleted by centrifugation at room temperature for 10 min at 400 *g*. The pellet is resuspended in 10 ml of 2.5% FCS-DMM and counted.

B. Cell Fusion and Selection of Hybrids

Although the fusion and the initial selection of hybrids by growth in HAT medium are quite distinct stages, they are described together for convenience. We will describe in detail procedures that follow the general scheme of Fig. 3. There are ways in which certain stages can be bypassed. These will generally be dealt with under the heading Other Procedures (Section IV,B,3).

1. *Fusion of Cells in Suspension*[5,6]

Materials

Sterile conical tube, 50-ml (Falcon, Cat. No. 2070)
Sterile conical tube, 10-ml (Sterilin, Cat. No. 144AS)
Water bath, 40°
Hot block, 37°
Beaker, 200-ml
50% PEG: Polyethylene glycol (10 g), MW 1500 (BDH Chemicals Ltd., Poole, Dorset, England, Cat. No. 29575) is autoclaved in a 25-ml glass tube. While still liquid 10 ml of warm (37°) sterile DMM are added, and the solution is thoroughly mixed, inverting the tube. The pH is checked by the color of the phenol red and adjusted to around 7 by leaving the tube open in a sterile hood or by blowing 10% CO_2–air mixture into the tube.
Sterile pipettes, 1-, 10-, and 25-ml capacity
Linbro 24 wells plates (Flow Laboratories, Cat. No. 76-033-05)
DMM, 200 ml

[5] G. Galfrè, S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard, *Nature (London)* 266, 550 (1977).
[6] G. Galfrè, C. Milstein, and B. W. Wright, *Nature (London)* 277, 131 (1979).

20% FCS–DMM and 2.5% FCS-DMM: 500 ml of DMM, 100 ml and 12 ml of FCS, respectively
HAT medium: 600 ml (see Section II,A)

Procedure

1. Parental cells are prepared as described above.
2. Mix 10^8 spleen cells and 10^7 (mouse) or 6×10^7 (rat) myeloma cells in a 50-ml conical tube; add DMM to a volume of 50 ml.
3. The cells are spun down at room temperature for 8 min at about 400 *g*.
4. The supernatant is removed with a Pasteur pipette connected to a vacuum line. Complete removal of the supernatant is essential to avoid dilution of PEG.
5. The pellet is broken by gently tapping the bottom of the tube. The tube is placed in a 200-ml beaker containing water at 40° and kept there during the fusion (steps 6–12). We do not consider it necessary to use a more cumbersome 37° water bath within the sterile cabinet.
6. Add 0.8 ml of 50% PEG prewarmed at 40° to the pellet using a 1-ml pipette, over a period of 1 min, continually stirring the cells with the pipette tip.
7. Stirring of the cells in 50% PEG is continued for a further 1.5–2 min. By then agglutination of cells must be evident.
8. With the same pipette, add 1 ml of DMM, taken from a tube containing 10 ml of DMM kept at 37° in the hot block, to the fusion mixture, continuously stirring as before, over a period of 1 min.
9. Repeat step 8.
10. Repeat step 8 twice, but add the medium in 30 sec.
11. Always with the same pipette and continuously stirring, add the rest of the 10 ml of DMM over a period of about 2 min.
12. With a 10-ml pipette add dropwise 12–13 ml of prewarmed DMM.
13. Spin down as in step 3.
14. Discard the supernatant, break the pellet by gently tapping the bottom of the tube, and resuspend in 49 ml of 20% FCS-DMM.
15. Distribute the fusion suspension in the 48 wells of two Linbro plates. These may contain a feeder layer of fibroblasts (see Section X).
16. Add a further 1 ml of 20% FCS-DMM. If a fibroblast feeder layer is not being used, add 10^5 spleen cells/ml (prepared as described in Section IV,A,1, step 9).
17. Incubate overnight at 37° in a CO_2 incubator.
18. With a Pasteur pipette connected to the vacuum line remove 1 ml

of culture medium from each cup without disturbing the cells that have settled in the bottom.

19. Feed the plate, adding 1 ml of HAT medium to each cup. Feeding with HAT medium is repeated for the next 2 or 3 days and after that once a week until vigorous growth of hybrids. This becomes evident under the microscope after day 10, but might take up to a month. At this stage the cultures become more yellow and are ready to be tested for antibody activity.
20. Duplicates of the growing hybrid cultures—either all or selected ones—are prepared and fed for a week with HT medium. Larger cultures can be prepared and frozen in liquid N_2. After a week in HT medium the cultures can be grown in the absence of HAT additives. Adaptation to lower concentration of serum can now be attempted.

2. *Filter Fusion*

The above procedure is not suitable for handling fewer than 4×10^7 spleen cells. For smaller numbers of cells we use a different protocol, essentially as described by Buttin *et al.*[7]

Materials

Filter fusion unit: We use the bottom half of a Millipore filtration set containing the mesh, the support, and the Teflon gasket. The top half holding the filter in position is replaced by a properly designed tube 3 cm long, made either of stainless steel or autoclavable plastic (see Fig. 4). The unit is fitted with a 25 mm 3.0 micropore size cellulose acetate filter (Millipore, S.A. Cat. No. SSWPO2500). The assembled unit is placed in an autoclavable centrifuge tube of appropriate size (e.g., M.S.E., Cat. No. 34411-166). The tube is closed, and the cap is held in position by a strip of autoclave tape.
Petri dishes: 3 cm diameter sterile (Sterilin, Cat. No. 301V); 4.5 cm diameter sterile (Sterilin, Cat. No. 302V)
Sterile forceps
Linbro 24 wells plate with feeder layer (see Section X)
Sterile pipettes: Pasteur, 10 and 25 ml capacity
Sterile tubes, 25 ml capacity
The following reagents as described above:
50% PEG, 2 ml
DMM, 5 ml

[7] G. Buttin, G. LeGuern, L. Phalente, E. C. C. Linn, L. Medrano, and P. A. Cazenave, *Curr. Top. Microbiol. Immunol.* **81**, 27 (1978).

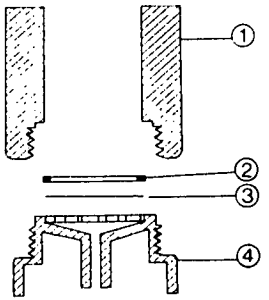

FIG. 4. Unit for filter fusions. The 3 cm-long tube (1) is of stainless steel 18/8 or Delrin (needs to be autoclaved and remachined a few times before use). The Teflon gasket (2) and filter support and mesh (4) are from a Swinnex-25 filter unit (Millipore S.A.); (3) is a cellulose acetate filter.

2.5% FCS-DMM, 20 ml
20% FCS-DMM, 50 ml
HAT medium

Procedure

1. Parental cells are prepared as described in Section IV,A.
2. Mix 10^6 spleen cells and 10^5 (mouse) or 6×10^5 (rat) myeloma cells and dilute to a total volume of 4 ml with DMM.
3. Open the tube containing the filter fusion unit and transfer the cell mixture to the unit.
4. Close the centrifuge tube and spin for 5 min at 400 *g* at room temperature.
5. Add 1.5 ml of warm (40°) 50% PEG to a 3-cm diameter petri dish.
6. After centrifugation open the centrifuge tube and, using sterile forceps, place the filter fusion unit in an open sterile petri dish (4.5 cm in diameter). No medium must be present on the filter.
7. Carefully dismantle the unit and remove the filter with sterile forceps.
8. Place the filter on top of the 50% PEG (step 5) with the cell layer facing down. Avoid bubbles under the filter. Incubate 1–3 min.
9. In the meantime transfer 5 ml of 20% FCS-DMM to a 4.5 cm diameter petri dish.
10. Remove the filter from the 50% PEG and place, cells facing down, in the 20% FCS-DMM.
11. Incubate overnight at 37° in a CO_2 incubator.
12. During the incubation most of the cells will detach from the filter

and settle on the bottom of the petri dish. Lift the filter with sterile forceps and wash the remaining adhering cells into the petri dish, using a Pasteur pipette and medium from the dish.

13. Using the same pipette transfer all the cells to a tube and add 20 ml of 20% FCS-DMM.
14. Distribute the cell suspension in the 24 wells of the previously prepared Linbro plate containing a feeder layer. Add 1 ml of HAT per well.
15. Incubate at 37° in a CO_2 incubator and proceed as described in Section IV,B,1 from step 18 on. Vigorous growth of hybrids will usually become discernible under the microscope after 2 weeks.

3. *Other Procedures*

Variations to the fusion protocol are described by several authors. The most important involve the addition of dimethyl sulfoxide to the polyethylene glycol,[8] changes in the concentration and molecular weight of polyethylene glycol as well as time of treatment,[9,10] centrifugation of cells on flat surfaces,[11] and variations in the ratio of spleen to myeloma cells. The choice of protocol does not seem to be critical, as all of them have been used successfully.

After fusion it is not necessary to fractionate the cell suspension as described in Section IV,B,1, step 15 on. Alternative procedures range from fractionation into a much greater number of microcultures, and in 200-μl well plates, to direct cloning onto semisolid agar.

4. *Controls*

Failure to grow hybrids after HAT selection is not uncommon on the introduction of the technique to a laboratory. This can be due to many reasons, but the first to be considered is the correct maintenance of the parental cells as discussed in Section III,B. Overgrown cultures will not recover in a few days, and frozen cultures are likely to take 2 weeks before they are in a suitable state for fusion. Poor media or accidental contamination with toxic substances is the second most common source of failure. The reagents and equipment used in the fusion and selection stages should be quality-controlled. For instance, the HAT medium should be controlled by growing established hybrids at low dilutions.

[8] T. H. Norwood, C. J. Zeigler, and G. M. Martin, *Som. Cell Gen.* **2**, 263 (1976).
[9] R. L. Davidson, K. A. O'Malley, and T. B. Wheeler, *Som. Cell Gen.* **2**, 271 (1976).
[10] V. L. Gefter, D. H. Margulies, and M. D. Scharff, *Som. Cell Gen.* **3**, 231 (1977).
[11] K. A. O'Malley and R. L. Davidson, *Som. Cell Gen.* **3**, 441 (1977).

Polyethylene glycol damages cells: overtreatment results in excessive cell death, undertreatment in insufficient fusion. The simplest check consists of careful examination of cells the day after fusion, before addition of HAT. The parental myeloma cells should show signs of growth. This can be most carefully controlled by limiting dilution analysis, comparing the growth in the absence of HAT of those cells treated and not treated with PEG.

Assays

The choice of assay used during the screening stages, to detect and clone the hybrid secreting the desired antibody, is of the utmost importance and should be given the greatest attention. Over the years immunologists have developed an enormous variety of ways of detecting the presence of antibodies, ranging from precipitation reactions and radioimmunoassays to assays based on the biological activities of the recognized antigens. Extensive reviews of such methods are to be found in other articles in this volume, in the "Handbook of Experimental Immunology,"[12] etc. But not all such assays are directly applicable to monoclonal antibodies. This is for two main reasons. First, the concentration of antibody in the tissue culture supernatant is usually much lower than that of a hyperimmune serum, and, second, traditional immunoassays often rely on the polyvalent recognition of antigens typically obtained with polyclonal antisera. Taking these factors into consideration, it is usually possible to adapt any immunoassay to detect monoclonal antibody in the supernatant of hybrid cultures. There are two general ways to detect the presence of antibody-secreting hybrids. The first utilizes the spent medium of growing cultures: the second directly detects the presence of antibody in the microenvironment of isolated cells or clones of cells grown in a semisolid medium.

V. Detection of Antibody in the Spent Medium

No method guarantees detection of all the clones secreting specific antibodies. For instance, not all immunoglobulin classes can fix complement, and therefore not all are detectable by direct lytic assay. This can be overcome by the addition of a second developing antibody (antiglobulin antibody, indirect lysis). However, the ratios of both antibodies are critical, and excess of either can inhibit lysis. The multiplicity of classes and subclasses makes it difficult to choose conditions that will ensure lysis for

[12] "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed. Blackwell, Oxford, 1978.

all of them. Indirect binding assays are more generally used for their simplicity, accuracy, versatility and ability to detect the largest proportion of antibody-secreting clones. But even they are limited by the specificity of the second antibody and by the number of binding sites.

A. Binding Assays

The insoluble antigen and the antibody in the culture fluid are allowed to react. The free antibody is washed away. The amount of monoclonal antibody bound is measured directly (direct binding assay) or by binding of a second, labeled antibody capable of recognizing the first (indirect binding assay). This second antibody can be labeled in several ways. We will describe two of the most commonly used. Others include, for instance, enzyme-linked derivatives,[13,14] or the use of protein A as an alternative to the second antibody.[15] It should be remembered that protein A does not bind to some classes (notably IgM) or even to some IgG subclasses of mouse and rat antibodies.

1. Insolubilization of Antigen

Antigens are often naturally insoluble (e.g., cell surface antigens). Others need to be rendered insoluble, and this can be conveniently done by attachment to plastic, e.g., microtiter plates (as described below) or polystyrene balls.[16] If antigens are small molecules, like haptens, they can be conjugated with proteins as a preliminary step.

Materials

Phosphate buffered saline (PBS): NaCl, 8.0 g/liter; KCl, 0.2 g/liter; Na_2HPO_4, 1.15 g/liter; KH_2PO_4, 0.2 g/liter
Protein antigen solution: 20–100 μg/ml in PBS containing 5 m*M* EDTA, 0.1% azide
BSA–PBS: 10% BSA in PBS (w/v)
Microtiter plates: flexible polyvinyl chloride microtitration plates, 96 round U wells (Cooke microtitre plate, Cat. No. 1-220-24)

Procedure

1. Dispense 50 μl of protein antigen solution in each well of a microtiter plate except for those that are to be used as controls.
2. Incubate at 4° overnight.

[13] S. Avrameas, *Int. Rev. Cytol.* **27**, 349 (1970).
[14] J. Enguall and P. Perlman, *Immunochemistry* **8**, 871 (1971).
[15] S. Jonsson and G. Kronvall, *Eur. J. Immunol.* **4**, 29 (1974).
[16] B. R. Ziola, M. T. Matikainen, and A. Salmi, *J. Immunol. Methods* **17**, 309 (1977).

3. Empty the wells. Often the same solution can be used two or three times for coating plates, but it needs to be tested.
4. Fill the plate with BSA–PBS and incubate at room temperature for 3–4 hr. At this stage it can be stored for 1 week at 4°.
5. Remove the BSA–PBS.

2. Preparation of Iodine-Labeled Second Antibody

Materials

PBS: see above
Sephadex G-50 fine: preswollen in PBS
Chloramine-T: 2 mg/ml in 0.3 *M* sodium phosphate buffer, pH 7.3, prepared *fresh* before use.
Tyrosine solution: saturated solution of tyrosine in H_2O
1% BSA–PBS: 10% BSA (w/v) in PBS, adjusted to pH 7.3
Protein solution: about 1 mg/ml in PBS
^{125}I-labeled solution: sodium iodide pH 7–11, 13–17 mCi per microgram of iodine (Radiochemical Centre, Amersham, England, Cat. No. IMS.30)
Disposable pipette, 5-ml (Falcon pipette 7543)
Glass wool, Parafilm, glass test tube
Hamilton syringe, 50-μl
Several Pasteur pipettes

Procedure

1. Cut the 5-ml disposable pipette a few centimeters above the gradation, plug the pipette tip with a small amount of glass wool, and place in a stand.
2. Fill to the 5-cm mark with Sephadex G-50 fine in PBS.
3. Run 3 or 4 ml of 1% BSA–PBS through the column.
4. Wash the column thoroughly with several milliliters of PBS, leaving about 0.5 ml above the Sephadex.
5. Seal both ends of the column with Parafilm until ready for use.
6. Using 50-μl Hamilton syringe, transfer to the glass tube 10 μl of ^{125}I-labeled solution. Add 10 μl of chloramine-T and, quickly, 20 μl of protein solution.
7. Mix well and incubate for 15 sec–2 min (varies from protein to protein). Add 25 μl of tyrosine, 50 μl of 1% BSA–PBS, and 200 μl of PBS; mix.
8. Using a Pasteur pipette, carefully apply the mixture to the column prepared in steps 1–6.
9. Load three times in succession 0.2 ml and, finally, 0.5 ml of PBS.

10. Discard effluent.
11. Load 1.5 ml of PBS.
12. Collect effluent containing the labeled protein. The column can be washed with PBS and used several times.
13. Dilute 10 μl of labeled protein solution in 1 ml of PBS and count 10 μl of this dilution in a gamma counter. In a standard preparation about 5×10^5 cpm/μl of column eluate would be expected.
14. Store at $-20°$ in small aliquots and use within 1–2 months.

3. *Indirect Binding Assay*[17,18]

Materials

PBS–10% FCS: PBS containing 10% FCS (or 1% BSA) and 0.1% NaN_3. Animal serum other than FCS can be used if it does not interfere with the assay.

^{125}I-labeled second antibody: prepared as above, adjusted to about 5×10^4 cpm/50 μl.

Microtiter plates: flexible polyvinyl chloride microtitration plates, 96 round U-wells (Cooke microtitre plate, Cat. No. 1-220-24)

Rotary plate shaker (optional): microshaker (Dynatech, Microtitre)

Centrifuge plate carrier: to spin plates in a refrigerated centrifuge when insoluble antigen (e.g., cells) not bound to the plates is used.

Hot wire cutter: a device formed by a rigid base (30 cm × 15 cm × 1 cm) with a tungsten wire across the middle of the base, kept stretched by a spring at a height of 8 mm from the base. An electric current from a variable rheostat is used to heat the wire to a proper temperature to cut the wells from the rest of the microtiter plate. This is done by sliding the plate along the base and slicing away the top part. Adhesive paper is stuck on the bottom of all the wells before they are cut out with the hot wire.

Multidispenser: multichannel reagent dispenser (Cooke Engineering Co.; available from Gibco-Europe, Cat. No. AM58)

Procedure

1. Antigen-coated microtiter plates are prepared as described in Section V,A,1. Alternatively, if cells or other particles are used as antigen, they are suspended in PBS–10% FCS. Apply to each well 50 μl of cell suspension containing between 5×10^5 and 6×10^6 cells.

[17] A. F. Williams, *Contemp. Top. Mol. Immunol.* 6, 83 (1977).

[18] L. A. Herzenberg and L. A. Herzenberg, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Chapter 12.22. Blackwell, Oxford, 1978.

2. Add 50 μl of spent culture medium that is to be tested (first antibody) to each well. A negative control must be included, using tissue culture medium. A positive control containing dilutions of the serum of the immune animals, or other antibodies previously obtained, is desirable.
3. Mix contents of wells (about 10 sec if using a rotary plate shaker).
4. Cover the plate and incubate at 4° for 45–60 min.
5. Wash the plate. (*a*) If antigen-coated plates are used, fill each well with 150 μl of PBS–10% FCS (a multidispenser can be used) and empty by inverting and vigorously shaking the plate over a sink (or a 1-liter beaker if radioactive material is to be discarded separately). Repeat the cycle twice. (*b*) To wash the wells containing cells, spin the plate 5 min at 400 *g* at 4°. Remove medium by suction. Shake the plate for 10 sec in the rotary shaker. Add 200 μl of PBS–10% FCS to each well. Repeat the whole cycle, spin as above, and remove medium by suction.
6. Add 50 μl of radioactive second antibody.
7. Cover the plate and shake for about 10 sec in the shaker.
8. Incubate at 4° for 45–60 min.
9. Wash plate as above, adding at least one extra cycle.
10. Dry the plate for 30 min in a 37° oven and cut the wells with a hot wire. Care should be taken to keep the wells attached to adhesive paper to keep them in order.
11. Using forceps, transfer each well into clean, labeled counting tubes. Count in a gamma counter.

An alternative possibility after step 9 is to add 50 μl of PBS–10% FCS to each well. The pellets are resuspended by shaking the plate; they are then transferred to clean tubes to be counted. If the antigen is immobilized on the plate, 50 μl of 1 *N* NaOH can be used to solubilize the material.

4. *Fluoresceinated Second Antibody*

This reagent is easily adaptable to qualitative screening, especially in combination with fluorescent microscopy[19] and cytofluorometry.[20]

Materials

Buffer: NaCl, 1.5 g/liter; Na_2CO_3, 1.95 g/liter; $NaHCO_3$, 2.66 g/liter; pH 9.3

[19] G. D. Johnson, E. J. Holbrow, and J. Dorling *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Chapter 15.16. Blackwell, Oxford, 1978.

[20] L. A. Herzenberg and L. A. Herzenberg, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Chapter 22.1. Blackwell, Oxford, 1978.

FITC solution: fluoresceinisothiocyanate (isomer I from Sigma, Cat. No. F7250), 1 mg/ml in buffer, pH 9.3
Protein solution: about 1 mg/ml in buffer pH 9.3
Sephadex G-50 column prepared exactly as described in the protocol for ^{125}I-labeling (steps 1–6) and equilibrated with PBS

Procedure

1. Add 0.3 ml of FITC solution to 1 ml of protein solution and incubate at room temperature for 3 hr.
2. Load onto the Sephadex G-50 column previously prepared.
3. Load three times in succession 0.2 ml and, finally, 0.5 ml of PBS.
4. Discard column effluent.
5. Load 1.5 ml of PBS.
6. Collect five fractions of about 0.3 ml.
7. Measure the optical density at 280 nm and at 495 nm. Pool the fractions, giving a ratio OD_{280}: OD_{495} of about 1. (Alternatively the fraction containing fluorescent protein can be identified under an ultraviolet light.)
8. Dilute with 1 volume of 10% BSA–PBS (0.1% NaN_3) and store at 4° or frozen in aliquots at −20°.
9. Dilute as appropriate before use.

5. *Preparation of Internally Labeled Antibody*

Unlike ordinary antibodies, monoclonal antibodies can be easily labeled internally at high specific activity, using radioactive amino acid precursors. The choice of these is based on the efficiency of incorporation of labeled amino acids into secreted immunoglobulin in culture conditions (Table II). We normally use radioactive lysine. Although the incorporation per amino acid residue is higher for arginine and phenylalanine, the number of lysine residues is usually higher. The more commonly used leucine is only about half as efficient as lysine. We reserve [^{35}S]Met or [^{35}S]Cys labeling for special uses. We routinely use [^{3}H]Lys for quantitative binding studies[6] and for immunocytochemistry.[21] For the chemical characterization of the monoclonal antibody (Section IX) it is simpler to use [^{14}C]Lys.

Materials

−Lys DMM: DMM without L-lysine (Gibco Bio-Cult)
Dialyzed FCS: fetal calf serum is dialyzed against double-distilled water. After dialysis, add 1/9th volume of 10 times balanced saline solution.
[^{3}H]Lys or [^{14}C]Lys: L-[4,5-^{3}H]lysine monohydrochloride, 5 mCi in 5 ml (The Radiochemical Centre, Amersham, England, Cat. No. TRK.520), or L-[U-^{14}C]lysine monohydrochloride, 250 μCi in 5 ml (Cat. No. CFB.69).
Incorporation medium: −Lys DMM, 9 ml; [^{14}C]Lys, 1 ml; dialyzed FCS, 0.5 ml or −Lys DMM, 2.5 ml; [^{3}H]Lys, 1.8 ml; 10 times balanced saline solution, 0.2 ml; dialyzed FCS, 0.5 ml

Procedure. About 2×10^6 cells from an exponentially growing culture are centrifuged, resuspended in −Lys DMM and pelleted by centrifugation. They are resuspended in 1 ml of incorporation medium and incubated at 37° in a water-saturated CO_2 incubator. Radioactive supernatant can be collected after 16–20 hr of incubation. Alternatively, after 8 hr of incubation a further 2×10^6 cells are washed as above and the pellet is

[21] A. C. Cuello, C. Milstein, and J. V. Priestley, *Brain Res. Bull.* **5**, 5 (1980).

TABLE II
EFFICIENCY OF INCORPORATION OF DIFFERENT AMINO ACIDS INTO MYELOMA PROTEIN MOPC 21 UNDER TISSUE CULTURE CONDITIONS[a,b]

Amino acid	Radioactivity[c] (%)	Radioactivity / residues[d]
Lys	20.1	3.2
His	1.6	0.6
Arg	15.5	5.5
Asp	0.6	0.06
Thr	11.2	1.1
Ser	4.2	0.3
Glu	1.3	0.1
Pro	7.6	0.8
Gly	3.6	0.5
Ala	0.5	0.1
Val	4.0	0.4
Met	0.9	0.4
Ile	3.2	1.0
Leu	8.2	1.4
Phe	17.3	4.1

[a] Unpublished data of J. Svasti and C. Milstein (1972).
[b] For this experiment the αα mixture was used and incorporation was measured after total hydrolysis of the purified IgG.
[c] Recovered after total hydrolysis.
[d] Refers to the number of moles of each residue per mole of protein after total hydrolysis.

added to the radioactive culture. The supernatant is collected after a further 10–12 hr of incubation.

For quantitative binding and immunocytochemical applications, purification is required to reduce the radioactive background. Often extensive dialysis is sufficient.[6] In other cases more extensive purification is required.[22]

6. *Inhibition of Direct Binding*

Among the quantitative binding studies, internally labeled monoclonal antibodies are particularly valuable to recognize other monoclonal antibodies with similar specificity. This is important for mapping antigenic determinants and detecting possible redundancy during the screening of hybrid cultures.[23,24] For these purposes we measure the inhibition of the direct binding of a labeled monoclonal antibody by the supernatants of the hybrids under study. The procedure is essentially as described in Section V,A,3 with the following modifications: (*a*) omission of step 5; (*b*) in step 6, the use of internally labeled monoclonal antibody instead of radioactive second antibody; (*c*) in step 11, transfer of each well to counting tubes and addition of 2 ml of Aquasol-2 (New England Nuclear, Cat. No. NEF-952) before counting. Quantitative inhibition studies require adequate titration of reagents.

Appropriate monoclonal anti-immunoglobulin antibodies can also be internally labeled and used as second antibody for indirect binding assays.

B. Hemagglutination Assays[25]

These assays are based on the ability of an antibody to agglutinate red cells carrying the specific antigen. They have all the advantages in terms of extreme simplicity, speed, and direct visual reading of results. The disadvantages are the inhibitory effects due to excess antibody (prozone effects) and quantitative inaccuracy. In practice these assays often fail to detect a number of antibody-secreting clones.

Inhibition of hemagglutination is a very simple way in which to define the specificity of the antibodies. This is done by simply adding excess antigen and antigen analogs to an appropriate dilution of hybrid supernatants before the addition of red blood cells.

[22] P. J. Lachmann, R. G. Oldroyd, C. Milstein, and B. W. Wright, *Immunology* **8**, 503 (1980).
[23] T. Springer, G. Galfrè, D. Secher, and C. Milstein, *Eur. J. Immunol.* **8**, 539 (1978).
[24] J. C. Howard, G. W. Butcher, G. Galfrè, C. Milstein, and C. P. Milstein, *Immunol. Rev.* **47**, 139 (1979).
[25] R. R. A. Coombs, *in* "Immunoassays for the 80s" (A. Voller, ed.), MTP Press, London, 1980.

1. *Attachment of Protein Antigens to Red Cells*

Materials

Red blood cells (RBC), usually from sheep
Saline: 0.9% NaCl in distilled water
$CrCl_3$ solution: 0.5 mg of $CrCl_3$ per milliliter in saline adjusted to about pH 5 by addition of NaOH, taking care to avoid the formation of any precipitate.
Protein antigen: about 1 mg/ml in saline. (Not PBS: phosphate inhibits $CrCl_3$ coupling.)
PBS, pH 7.2

Procedure

1. Wash the RBC three or four times in saline.
2. In a round-bottom tube containing 1 volume of packed RBC, add 1 volume of $CrCl_3$ solution and 1 volume of protein antigen solution. The two solutions should be added simultaneously, using two pipettes.
3. Immediately resuspend the cells by inverting the tube several times; continue this for 2 min.
4. Add at least 10 volumes of PBS; mix by inversion, and spin down at 1000 *g* for 5 min.
5. Repeat the wash three times and resuspend the coated RBS in PBS. Sterile coated RBC can be stored for several weeks at 4°.

2. *Direct Hemagglutination*

In each well of a microtiter plate (round-bottom U-wells) dispense 25 μl of RBC–PBS (1 : 16, v/v). Add 25 μl of supernatant to be tested and mix well using a plate shaker. Incubate at room temperature for 2 hr. Agglutinated RBC fail to settle as a tight pellet. The plate can be photographed. For a more accurate reading, the pellet of each well is carefully transferred onto a microscope slide. Microscopic examination can detect very weak agglutination.

3. *Indirect Hemagglutination*

At the end of the direct agglutination test it is possible to add a titrated amount of anti-immunoglobulin to each well. The pellets are then resuspended and allowed to settle for a further 2 hr. After this period, results can be recorded as above. Better, but more time-consuming, is to remove the first antibody before the addition of the second. The second antibody must be tested before use. It must not agglutinate coated RBC in the absence of the first antibody at the concentration used in the final test.

C. Lytic Assays

These assays are based on lysis of cells by antibody and complement. The extent of cell lysis can be measured in several ways. One is the release of ^{51}Cr incorporated into target cells carrying the desired antigen. This and other related methods are particularly applicable to cell surface antigens.[26,27] We will describe another method that is based on visual observation of lysis of red cells.[28] This is of general application. Soluble antigens can be attached to the red cell surface as is done for hemagglutination assays.

Spot Tests

Materials

Agarose 0.6%: 6 g of indubiose A37 (l'Industrie Biologique Française) dissolved in 100 ml of PBS by boiling for at least 10 min

Coated RBC: Prepare as described in Section V;B,1. Use as 1 : 4 (v/v) suspension in PBS.

Monoclonal antibody: If tissue culture supernatants are to be tested add one drop of 5% NaN_3, 1.2 *M* HEPES to 1 ml of spent medium.

Developing antibody: Anti-rat or mouse immunoglobulin antiserum. The developing antibody should not lyse coated RBC and should be titrated in a spot test procedure similar to the one to be used: excess causes inhibition of lysis.

Guinea pig complement (GPC'): Blood from normal adult guinea pigs is allowed to clot at 37° for 1 hr. Clarify the serum by centrifugation at 1500 *g* for 20 min at 4°. To 9 volumes of serum add 1 volume of 0.1 *M* EDTA in PBS and 3 volumes of packed RBC. Incubate for at least 1 hr at 4° with continuous mixing by inversion of the tube (about 1 inversion per second). Add 1 volume of $CaCl_2$, 0.1 *M* in PBS. Centrifuge for 10 min at 1500 *g* at 4°. The GPC' should be aliquoted and stored at −70° or, preferably, in liquid N_2. Complement activity is easily lost by freezing and thawing. Thawing is done at 37° with mixing.

Petri dishes: The procedures given below apply to plastic petri dishes 9 cm in diameter. Different sizes can be used, adjusting the reagent volumes. It is advisable, particularly if glass petri dishes or small plastic ones are to be used, to coat them by pouring a base of 7 ml of 1.5% agarose in PBS in each 9-cm dish. These can be stored inverted in a humid chamber at 4° for several weeks. Before use, a reticulate is drawn with a Magic Marker on the base of the dish, dividing it into approximately 20–30 identifiable areas.

Glass tubes: The convenient size to hold 2 ml of agarose is about 5 cm long, 10 mm in diameter, round-bottomed and rimless.

[26] H. S. Goodman, *Nature (London)* **190**, 269 (1961).
[27] T. Pearson, G. Galfrè, A. Ziegler, and C. Milstein, *Eur. J. Immunol.* **7**, 684 (1977).
[28] N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963).

Quick Mix Procedure

1. In a glass tube kept at 42° add 2 ml of 0.6% agarose, 100 μl of RBC, 200 μl of GPC', and 100 μl of developing antibody at an appropriate dilution.
2. Mix well by rotating the glass tube between the hands and pour immediately onto the petri dish to form an even layer. Let the agarose set for 5–10 min.
3. Spot onto the marked area of each petri dish 3–5 μl of the spent medium to be tested. Cover with the lid and incubate at 37° in a humid chamber. Lytic areas are generally evident after 1 hr, but longer incubations may be required. Lysis can best be seen against a dark background and with lateral illumination.

Two-Step Procedure

1. To 2 ml of 0.6% agarose kept in a glass tube at 42° add 100 μl of RBC.
2. Mix well by rotating the tube between the hands and pour to form an even layer on the petri dish. Set for 5–10 min.
3. Spot 3–5 μl of the supernatants to be tested. Allow the drops to dry, leaving the dish open, for 5 min. Cover and incubate at 37° in a humid chamber for 1 hr.
4. Pour into each dish 3 ml of a solution containing 10% GPC' and a titrated amount of developing antibody.
5. Incubate at 37° in a humid chamber. Lytic areas are generally evident at 1 hr, but it is advisable to incubate for at least 4–6 hr before giving results a negative score.

D. Assays Based on Biological Activity of Antigen

Antibodies can be recognized by their effect on the biological activity of an antigenic substance. The simplest way is to add individual culture supernatants to a biologically active preparation of antigen (e.g., an impure interferon preparation[29]). After a suitable period of incubation, a decrease in biological activity is taken as preliminary evidence for the

[29] D. S. Secher and D. C. Burke, *Nature (London)* **285**, 446 (1980).

presence of inhibitory antibody. On the other hand, the precipitation of antigen–antibody complexes can be effected by different procedures; for instance, by addition of carrier mouse or rat immunoglobulin and anti-mouse or rat immunoglobulin to equivalence. Alternatively, anti-rat or anti-mouse immunoglobulin or protein A attached to Sepharose can be used to absorb the antigen–antibody complexes.[30] The important aspect of these methods is that they afford an exquisite specificity of recognition without the need for antigen purification.

VI. Direct Detection of Antibody-Secreting Cells

Direct identification of antibody-producing cells relies on detecting the minute amount of antibody present on the cell surface or in the immediate vicinity of the cells immobilized on semisolid medium.

Antibody-secreting cells, under appropriate conditions, bind a certain amount of antigen. It has been possible to detect cells secreting specific antibody by attaching the antigen to fluorescent microspheres. A method has recently been described whereby cells in suspension are rendered fluorescent in this way and are automatically separated from the rest by a fluorescent-activated cell sorter.[31]

When cells are grown on a semisolid support of agar or agarose, the antibody secreted by the cells diffuses slowly, and methods have been developed to visualize it, either *in situ*[1,32] or by replica methods. The replica immunoadsorption method[33] is based on the adsorption of the antibody secreted by the clones onto nitrocellulose filters that have been precoated with antigen or anti-immunoglobulin. The filters are placed on the surface of the agarose containing the growing hybrid clones. After a suitable time, the filter is removed and the presence of localized areas containing specific antibody can be revealed by the binding of labeled antigen. For instance, a suspension of antigen-coupled erythrocytes is overlaid and the unbound erythrocytes washed away. Red spots delineate the sites at which antibody-forming clones are present in the agarose.

A. Plaque-Forming Clones

We describe here in detail a method[34] for the direct visualization of antibody-secreting clones based on the complement-dependent, localized

[30] T. Pearson and L. Anderson, *Anal. Biochem.* **101**, 377 (1980).
[31] R. Parks, V. M. Bryan, V. T. Oi, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1962 (1979).
[32] N. K. Jerne, C. Henry, A. A. Nordin, H. Fuji, A. M. C. Koros, and I. Lefkovits, *Transplant. Rev.* **18**, 130 (1974).
[33] J. Sharon, S. L. Morrison, and E. A. Kabat, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1420 (1979).
[34] C. Milstein and B. W. Wright, unpublished data, 1979.

lysis of antigen-coated red blood cells. The optical properties of sheep RBC allow easy visualization of local areas of lysis around antibody-secreting clones. The lysis of other types of target cells can also be used to localize antibody, but then live and dead target cells are visualized by vital strains.[35] The method has been applied to the detection and isolation of clones secreting antibodies to cell surface antigens.[36]

Materials

Agarose, 1.2% (w/v): The quality of the agarose is critical. It often has anticomplement activity. From this point of view, indubiose A37 is best, but we have been unsuccessful in using it for cloning (although it is best for overlays; see below). Some batches of LGT agarose (Marine Colloids, Inc.) are appropriate for both: preliminary tests, using it for spot tests (as described in Section V,C), are recommended for new batches. Agarose is suspended in tissue culture grade distilled water, autoclaved, and kept at 42°.

Concentrated FCS-DMM: 500 ml of 2 × DMM (Section II,A); 20 ml of penicillin-streptomycin, 5000 units/ml 200 ml of FCS; and 10 ml of 100 m*M* sodium pyruvate

Agarose, 0.5%: 1 volume of 1.2% agarose and 1.2 volume of concentrated FCS-DMM. Keep at 42°.

Cells: A vigorously growing culture should be used. Wash cells and prepare suspensions in 20% FCS-DMM containing appropriate cell dilutions (e.g., 1000, 5000, and 25,000 cells/ml). Keep at 37°.

Linbro plates: 6-dish Linbro plate (Flow Laboratories, Cat. No. FB-6-TC)

Coupled SRBC (1:4 in PBS), guinea pig complement suitably absorbed and developing second antibody (optional) are as described in Section V,C.

Preparations of Base Layers. Use at least one 6-dish plate for each culture to be cloned. The dishes should preferably be seeded with a feeder layer 24 hr in advance. Remove all the medium and apply 2 ml of 0.5% agarose to every dish. Set at 4° on a level surface.

Cloning. Take 150 μl of 0.5% agarose and add 135 μl of each cell suspension and 15 μl of coupled SRBC. Apply dropwise and as evenly as possible to the top of the cold agarose base layers. Prepare duplicates using 150 μl of 0.5% agarose and 150 μl of cell suspension, but no SRBC. Controls with 20% FCS-DMM substituting the cell suspension should also be carried out to test the stability of red cells in the absence of hybrids. Put plates back into refrigerator; keep level for about 15 min to

[35] H. Fuji, M. Zaleski, and F. Milgrom, *J. Immunol.* **106**, 56 (1971).
[36] P. Lake, E. A. Clark, M. Khorshidi, and G. M. Sunshine, *Eur. J. Immunol.* **9**, 875 (1979).

solidify top layer. Transfer to 37° in CO_2 incubators. Check for growth after 48 hr.

Revealing Clone Plaques

1. The cultures containing the coupled SRBC should be tested at about 48 hr. Densely growing cultures could result in complete lysis. Add to each well 0.3 ml of 10% FCS–DMM containing 10% GPC' (and developing antiglobulin antibody at the appropriate concentration when required). Replace plates in the incubator. Observe under dark field after 2 hr, 4 hr, and overnight. A stereomicroscope with low magnification is very useful for this purpose. Areas of lysis (plaques) should appear around antibody-producing clones. These are allowed to grow for 7–10 days before picking them.

2. The wells that do not contain red cells can be left for about 7 days, and clone plaques can be revealed by an overlay procedure.[1] Add to each well 0.3 ml of a suspension made up of 2 ml of 0.5% agarose, 0.2 ml of GPC' and 0.1 ml of coated SRBC. Addition of anti-mouse immunoglobulin antibody is also recommended. The amount to be used should first be titrated as it is inhibitory in excess. Lytic areas around clones usually appear within 2 hr at 37°.

Variations in the order of addition of the reagents can have dramatic effects on the results. For instance, the agarose overlay containing the red cells can be applied first. After incubation for 2 hr, a 0.3 ml solution containing the guinea pig complement and the second antibody is added. Lytic areas should appear on further incubation at 37°. This two-step method allows the monoclonal antibody to bind to the red cells before the antiglobulin reacts with it and is more sensitive. At first sight this appears less risky, but this is not so. The problem is much more complex, and in some cases rings of lysis are observed. This is because the essential requirement for lysis is the formation of an aggregate of monoclonal antibody and anti-immunoglobulin at the surface of the red cell. Excess of either of the two antibodies is inhibitory. The concentration of monoclonal antibody decreases as the distance from the clone increases. The rings of lysis appear at the point at which both antibodies are at equivalence.[37]

VII. Cloning

The timing of the cloning requires careful consideration. As a general rule it is best to clone as early as possible. Multiple clones in a single culture compete for growth and this, together with chromosome segregation, conspires against stability of expression (see Section VIII). However, hybrid lines are easier to clone after some time of active growth. It is possible to clone immediately after fusion (step 14, Section IV,B,1) without prior fractionation on Linbro plates. This is not recommended unless a method of direct detection of antibody-secreting clones is being used (Section VI).

[37] C. D. Wilde, Ph.D. Dissertation, Cambridge Univ. Library, 1979.

If the standard protocol in Fig. 3 is followed, supernatants from the microcultures at step 19 of Section IV,B,1 have been assayed. The cloning strategy somewhat depends on the number of independent positive cultures.

1. If only a few cultures are positive, it is worthwhile to subdivide them by limiting dilutions (see below), preferably in the presence of a feeder layer, and at the same time subject them to a cloning procedure.
2. If there are too many positive cultures to be conveniently handled in this way, duplicates should be prepared (to minimize the risk of accidental loss) and cell stocks be frozen in liquid N_2.

It is important at this stage to attempt to assess the interest of the different antibodies. This assessment must give priority to the use for which the monoclonal antibody is intended. There are two aspects of the antibody properties to be assessed. One relates to the antigenic recognition, and the other to the functional properties of the antibody. Antigenic recognition includes cross-reactive patterns, antigenic distribution on natural carriers, and fine specificity. Other functional properties include kinetic and thermodynamic parameters of antigen–antibody interactions, cytotoxicity, agglutination, effect of the antibody on the biological activity of antigens. In this analysis it must be kept in mind that, at this stage, supernatants may contain multiple antibody species.

When individual cultures are identified as of special interest they can be treated as in item 1 above. It may be that no special preference can be attached to individual cultures, in which case they are probably best left growing and more frozen stocks prepared. Instability and clonal competition will simplify the problem. Supernatants should be tested at regular intervals. Some cultures will gradually become negative, and the more resilient ones are those that will be easiest to clone and to handle.

If at any stage it is found that interesting clones have been lost, attempts to recover them can be made using the frozen stocks. In this event only stocks prepared well before the culture became negative should be used. It must be remembered that antibody can still be present when antibody-producing cells are no longer growing.

1. Limiting Dilution Fractionation

About 3×10^5 cells are transferred to the first cup of a 24-well Linbro plate containing a feeder layer. After thorough mixing a twofold dilution series is prepared over the first 12 cups (maximum dilution of about 600 cells in cup 12) or over all 24 cups (about one cell in cup 21 or 22 and none in cups 23 and 24). Part of the medium is changed every 4 or 5 days. Supernatants are collected and tested when the cultures approach confluence. The positive culture containing the minimum number of seeded cells can either be fractionated again as above or cloned in semisolid medium as soon as possible. Many variations on this basic protocol can be made. A common one is to use 96-well microtiter plates.

The positive cultures selected in this way should not be regarded as monoclonal. Correct cloning as described below should be performed at least once, and preferably twice.

2. Cloning on Semisolid Supports

Materials

2 × DMM–20% FCS: 100 ml of 2 × DMM from dry powder, 40 ml of FCS

1% agar: 1 g of Agar (Bacto Agar, Cat. No. 0140-01, Difco Laboratories) in 100 ml of tissue culture grade distilled water. Autoclave for 15–20 min. Keep at 42°.

0.5% agar: 1 volume of 1% agar, 1 volume of 2 × DMM–20% FCS. Keep at 42°. [If 10 × DMM is to be used, the 0.5% agar is better prepared by mixing 1 volume of 5% agar in water with 1 volume of 2 × DMM–20% FCS (prepared from the 10 × DMM), and 8 volumes of 20% FCS–DMM (prepared from the 1 × DMM).]

HAT or other additives as required

Petri dishes: 9 cm in diameter plastic, tissue culture grade (Sterilin, Cat. No. 304V).

Procedure. Pour into each petri dish (with or without a feeder layer) about 15 ml of 0.5% agar. Set for about 15 min at room temperature. Prepare several cell suspensions containing, for instance, 100, 500, 5000, and 50,000 cells/ml. Add 1 ml of 0.5% agar (at 42°) to 1 ml of each cell suspension (at room temperature). Mix by rotating the tube between the hands and pour immediately onto the agar base. Allow to set for at least 10 min at room temperature and incubate at 37° in a CO_2 incubator.

Clones can be picked at days 4–5 using a dissection microscope or, after 7–10 days, directly, and transferred to individual cups of a 24-well Linbro plate. It may be essential to have feeder cells in the cups, especially if small clones are picked. A clone should not be considered pure until it has been recovered from a plate grown at low density.

3. Cloning by Limiting Dilution

This is performed as described in Section VII,1 except that a single dilution is used so that, at most, only one cell is present in each microculture well. A fluorescent-activated cell sorter with a cloning attachment[37] is very convenient for this purpose; otherwise we prefer cloning on semisolid supports.

VIII. Selection of Positive Clones

Positive clones growing on semisolid supports can be identified by direct detection methods (see Section VI). From those plates containing the lower number of growing clones, several (at least six) should be picked when they are about 100–1000 cells. They are then transferred for further growth (Section VII,2).

If direct detection of positives is not possible, clones are picked randomly. The number of clones to be transferred for further growth depends on the expected frequency of positive clones. An informed guess is based essentially on the history of the antibody titer of the hybrid culture. Large numbers of random clones should be picked when the antibody titer decreases with time of culture. Care should be taken to pick small clones as well as large ones. On the other hand, if consistent or increasing titers are being obtained over a period of weeks or months, a random collection of 24 clones is a convenient number. The picked clones are allowed to grow to confluence, and the supernatant is assayed for antibody activity.

If none of the picked clones is positive, the most probable explanation is the presence of more vigorous negative competing clone(s). These can be variant clones that have lost the ability to secrete complete immunoglobulin (Fig. 2). If this is the case, clones that are positive for immunoglobulin secretion can be detected by direct methods, even if present at very low frequencies. This is done by a reverse-plaque method.[38] The detailed protocol is as described in Section VI,A. The red cells are coated with anti-immunoglobulin antibody that has previously been purified by affinity chromatography. Alternatively the red cells can be coated with protein A.[39] An *in situ* precipitation method can also be used that does not rely on lysis of red cells.[40] The plaque-forming clones secrete immunoglobulin but not necessarily the specific antibody. Such clones should be randomly picked and assayed for specific antibody.[29]

A simpler means of concentrating the specific antibody-producing clone can also be attempted by limiting dilution fractionation as described

[38] G. Köhler, S. C. Howe, and C. Milstein, *Eur. J. Immunol.* **6**, 292 (1976).
[39] E. Gronowicz, A. Coutinho, and F. Melchers, *Eur. J. Immunol.* **6**, 588 (1976).
[40] P. Coffino and M. D. Scharff, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 219 (1971).

above. But if this is to be done it may be much better to use stocks of cells frozen at earlier stages. This can become a rather tiresome exercise involving hundreds of microcultures.[41] It is only justified when chasing particularly valuable specificities.

Sometimes cultures remain positive for long periods, yet the clones are invariably negative. This may be due to cloning conditions selecting against the positive clone. The best course of action is modification of cloning conditions and methods.

IX. Derivation of Variants

From the collection of positive clones derived from each hybrid culture, at least three random ones should be grown up and frozen stocks prepared as a precautionary measure. Parallel to this an analysis should be made to explore the possible presence of individual clones producing different antibodies and of clonal variants. Sometimes the assay of the antibody activity of the supernatant will give either a clue to or a strong indication of clonal differences. Otherwise a biochemical analysis of the antibody secreted is very useful. Several tests can be made but a simple one is based on labeling the secreted products with radioactive amino acids (see Section V,A,5) and subsequent electrophoretic analysis. The radioactive supernatants are directly analyzed by sodium dodecyl sulfate gel electrophoresis[42] in the presence of reducing agents. Preliminary dialysis is unnecessary. The antibody can also be analyzed by isoelectric focusing. Full details of the apparatus and procedures used to analyze a large number of samples are given by Secher *et al.*[43] Intact IgM penetrates the acrylamide gel only under special conditions.[44] Isoelectric focusing of separated chains is described by Köhler and Milstein.[1] It is essential to include control samples on the electrophoretic plates to permit easy interpretation of results. This analysis gives a description of the chain composition of the antibody and distinguishes the γ and μ classes of heavy chain. Depending on the choice of parental myeloma line used for fusions, segregants that have lost the expression of the myeloma chains may be detected, and stocks from these should be frozen separately.

On the basis of the above analysis, of the growth characteristics of each clone, and of the stability of the antibody titer of the confluent supernatants, individual clones are transferred to bottles for the preparation of frozen stocks. Larger amounts of antibody can now be prepared,

[41] A. F. Williams, G. Galfrè, and C. Milstein, *Cell* 12, 663 (1977).
[42] U. K. Laemmli and M. Faure, *J. Mol. Biol.* 80, 575 (1973).
[43] D. S. Secher, C. Milstein, and K. Adetugbo, *Immunol. Rev.* 36, 51 (1977).
[44] A. Ziegler and H. Hengartner, *Eur. J. Immunol.* 7, 690 (1977).

but at the same time we consider it to be essential to reclone the line to ensure monoclonality and to achieve better stability of production. Even the best cloning technique cannot totally exclude the possibility of cross-contamination. A second cloning step performed at low cell density provides a fail-safe device.

It is better to use cultures that have been growing for a certain time for the second cloning. This is because for several months after fusion dividing hybrids tend to lose chromosomes and to attain a more stable genotype. It is therefore convenient to allow a certain amount of drift to facilitate the selection of a subclone that will have the best stability properties and the desired chain composition. The derivation of subclones follows the protocol described in Section VII. The number of subclones to be collected is based on the considerations discussed in Section VIII, except that when dealing with stable lines fewer subclones can be picked. However, if chain loss variants are sought many more subclones must be screened.

Procedure for the Derivation of Chain Loss Variants

1. It is preferable to use a culture that has not been recloned and that has been in continuous growth for a reasonable period (a month or longer).
2. Prepare clones as described in Section VII,2.
3. With a Pasteur pipette suck up a plug of agar containing a single large (at least 1000 cells) clone and blow the agar plug into a well of a microtiter plate containing 150 μl of incorporation medium (Section V,A,5). A few cells remain in the pipette, and these are carefully washed into a well of a second microtiter plate containing 150 μl of 20% FCS–DMM, with or without a feeder layer.
4. Repeat this procedure with at least 48 clones.
5. Put both plates in a humid CO_2 incubator.
6. After at least 16 hr of incubation, centrifuge the plate containing the radioactive samples for 5 min at 400 g. Transfer the supernatant to the empty wells of the plate or to another plate.
7. Analyze the radioactive supernatant by the electrophoretic method of choice.
8. If variants are identified, the culture contained in the replica plate is transferred into a larger culture dish.
9. Recloning of the selected variant should be performed as soon as possible because of the high risk of cross-contamination introduced by the requirement of large clones at step 3.

A procedure based on anti-idiotypic antiserum has been described.[45]

[45] T. Springer, *J. Immunol. Methods* (in press).

X. Feeders

There is no doubt that the presence of a feeder layer increases the ability of cultured cells to grow at very low densities. The use of feeders is therefore essential for isolating hybrid clones that are otherwise difficult to grow. It increases the yield of viable hybrids after the fusion step and is strongly recommended in the fusion protocol (Section IV,B,1) and is essential for the protocol of Section IV,B,2. It is also essential when cloning by limiting dilution. However the indiscriminate use of feeders introduces an often unnecessary complication. Furthermore, as the final aim of the overall protocol is the preparation of cloned hybrid lines that will grow vigorously in the least demanding culture media, we prefer to avoid the use of feeders as soon as this is possible.

For the fusion itself the simplest, although not necessarily the best, feeder is the same cell as used for the fusion. Different workers have recommended other normal cells, notably thymocytes and macrophages. It is objectionable and often less convenient to use cells from specially sacrificed animals. Feeders made from irradiated fibroblasts are a good alternative. Many different fibroblast lines can be used, and we have obtained reasonable results with the 3T3 mouse line obtainable from most tissue culture collections and suppliers.

Procedure. A large culture of fibroblasts is harvested in the logarithmic phase of growth, washed by centrifugation and irradiated with about 10,000 rad. After irradiation the cells are resuspended in freezing medium and frozen (see Section XI) in aliquots of about 5×10^5 cells/0.5 ml. Titration and control of each batch prepared is necessary. A vial is thawed, and from this twofold dilution cultures are prepared in a 24-well Linbro plate. After 3–4 days of culture, the well giving a 50% confluent monolayer is used to calculate the number of wells that can be prepared from each frozen vial. The dilution plate is kept for a further 7–10 days to check that no further growth is evident.

XI. Freezing of Cells

Many methods for freezing viable cells have been described, and some rely on fairly sophisticated apparatus to provide programmed temperature decrease. The method we will describe does not rank high in terms of recovery of viable cells, but it is extremely simple and ideally suited to the specific needs of derivation of hybrid myelomas. We find it very reliable provided that the cell stock used for freezing is in full logarithmic growth.

Freezing Procedure. About 10^6 to 10^7 cells are pelleted by centrifugation at 400 *g* at 4° for 7 min. The supernatant is removed, and the pellet is resuspended in 0.5 ml of freezing medium (9 parts FCS, 1 part dimethyl sulfoxide) at 4°. The suspension is transferred to a freezing vial (Sterilin, Cat. No. 506), and this is placed in a small insulating box (1 cm thick expanded polystyrene is adequate) and put at −70° for at least 20 hr. The vial is then transferred directly to liquid N_2.

Thawing Procedure. Thaw the vials as quickly as possible in a 37° water bath. When thawing is nearly complete, transfer the cell suspension to a 10-ml centrifuge tube in an ice bath. Slowly add 10 ml of cold 10% FCS–DMM, mixing carefully. Centrifuge at 400 *g* at 4° for 7 min. Resuspend the cells in about 5 ml of fresh medium, and transfer to a small tissue culture flask. It is better, but more laborious, to resuspend the cells in 2 ml and prepare a series of twofold dilution cultures in Linbro plates, with or without feeders.

XII. Large-Scale Production of Monoclonal Antibody

Large amounts of monoclonal antibody can be produced either by culturing cells *in vitro* or growing them as tumors *in vivo*. The monoclonal antibody is secreted and is accumulated in the spent medium of the cultured cells and in the serum and body fluids of the tumor-bearing animals. The two methods of production are complementary, as both have advantages and disadvantages. The concentration of monoclonal antibody in the spent medium is of the order of 10 μg/ml but can be increased to perhaps 50 μg/ml or even 100 μg/ml. The concentration of monoclonal antibody in the serum of a tumor-bearing animal is often about 10 mg/ml and may reach 3 or 4 times that value. The animal serum is therefore usually 1000 times more concentrated. But this is not always so. It has been observed that certain macroglobulins never reach high concentrations in the serum. The reasons for this are not clear, but it seems that some macroglobulins have a higher catabolic rate, preventing their accumulation in the serum. In one example the anti-blood group A activity of a monoclonal antibody taken from the serum was of a lower quality than the equivalent product taken from tissue culture. This was interpreted as being due to partial proteolytic degradation.[46]

The protein impurities present in the spent medium can largely be controlled because most come as components of medium. In particular the monoclonal antibody is the only immunoglobulin of rat (or mouse) origin in the spent medium. In contrast, serum from tumor-bearing animals always contains immunoglobulin impurities that are of the same species. Although such animals have a severe depletion of their normal

[46] D. Voak and C. Milstein, unpublished data, 1979.

immunoglobulin components, the antibody is not likely to be much better than 90% of the pure monoclonal variety. Tissue culture material is therefore intrinsically better as a source of monoclonal antibody. It is to be preferred when concentration is well above that required.

The high concentration of monoclonal antibody in the fluids of tumor-bearing animals makes them better for the preparation of chemically purified antibody. The purification protocol has to be adapted to each individual case, depending mainly on the antibody class. Usually a 50% ammonium sulfate precipitate gives better than 50% pure monoclonal antibody. Further purifications (for instance DEAE-column chromatography) are widely discussed in the literature. In the long run, even large-scale preparations of pure monoclonal antibody may use spent medium from cultured cells as a more humane and better controlled source. But this will depend on the technological development of large-scale cell culture methods.

A. Production in Culture

Before large-scale growth it is advisable to adapt the chosen clone to medium containing a low percentage of serum. This is usually achieved by feeding a vigorously growing culture with 5% FCS–DMM.

For Small Quantities. Transfer 20 ml of cells from above to a tissue culture bottle (800 ml, Flask Nunclon-Delta, Cat. No. N-1475, Nunc, Denmark) and dilute to about 50 ml with 2.5% FCS–DMM. Gas with 10% CO_2–90% air. Close bottle tightly and keep it in a dry incubator at 37°. After 1–2 days add a further 150 ml of 2.5% FCS–DMM and let the culture grow for a further 2 days or more until it has been in stationary phase at least one day. Collect the supernatant by centrifugation.

For Medium Quantities. Transfer 30 ml of a vigorously growing culture into a roller flask (850 cm^2 Roller Bottles, Falcon, No. 3027) and add 70 ml of 2.5% FCS–DMM. Gas as above. Close bottle tightly and keep it standing at 37° in a dry incubator. After 1–2 days add 700 ml of 2.5% FCS–DMM, close tightly, and keep rolling (1 rpm) at 37°. Open the bottle daily for 5–10 min in a sterile hood to allow gas exchange. Harvest the supernatant at least 1 day after cells have reached the stationary phase of growth.

For Larger Quantities. Transfer 200 ml of a vigorously growing culture to a 5-liter spinner (Fig. 1), dilute it with 200 ml of 5% FCS–DMM, and check for growth after 24 hr. If growth is vigorous, start diluting the culture with 2.5% FCS–DMM at a rate that will keep the culture in logarithmic growth. When the spinner is full, allow it to achieve stationary phase, leave it for a further 1–2 days, and harvest. Checks of antibody titers can be used as an indicator of the best harvesting time.

If even larger volumes are required a series of spinners can be organized in such a way that one spinner is kept permanently in logarithmic growth with 5% FCS–DMM and the others are used to dilute with 2.5% FCS–DMM.

Serumless Preparations. For synthetic media that do not include serum some formulations have been proposed.[47] But ordinary DMM (with no serum) can also be used. Prepare a vigorously growing culture in 5% FCS–DMM. Centrifuge cells and resuspend at a density of 1 to 4 × 10^6 cells/ml in DMM. Gas the flask. Incubate at 37° for 24–48 hr. Harvest.

Concentration of Antibody from Spent Medium

Spent medium can be concentrated using ultrafiltration devices (e.g., Minicon Concentrator, B15, Amicon Corporation). For larger volumes we prefer the following procedure.

Add solid $(NH_4)_2SO_4$ with gentle stirring to 50% saturation. Allow to equilibrate for at least 30 min. Centrifuge. Dissolve the precipitate with PBS (or alternative saline solution) using a volume of about 1/100 of the original spent medium. Dialyze against the chosen saline solution and clarify by centrifugation. The procedure is best carried out at 4°. Some monoclonal antibodies may be unstable to this treatment.

B. Production in Animals

Tumors can be derived as either solid or ascitic. Solid tumors are somewhat easier to derive and to manage, but the yield of antibody is usually higher from ascitic fluid. Animals should be histocompatible with the hybrid clone to be injected. For instance, a clone made with myeloma X63 (BALB/c origin) and spleen cells from a C3H mouse should be injected into (BALB/c × C3H) F_1 hybrids. Partial mismatching is sometimes acceptable but may require immunosuppressive treatment. In any event partial immunosuppressive treatment is often recommended for faster tumor growth. In some cases this is essential even with fully histocompatible combinations, possibly owing to somatic drift of tumor antigens. Adequate immunosuppression is usually achieved with a relatively low X-ray irradiation dose (say 500 rads) and/or an injection of about 0.5 mg of cyclophosphamide/20 g animal weight 24 hr before tumor transplantation. The use of drastic immunosuppression or immunodeficient strains of animals (e.g., nude mice) has been recommended for the growth of totally histoincompatible tumors, such as clones derived from a mouse myeloma and spleen cells from rats. We did not find that this procedure

[47] N. N. Iscove and F. Melchers, *J. Exp. Med.* 147, 923 (1978).

yielded better material than we could prepare by concentrating spent medium.

Solid Tumors. Cells taken from a vigorously growing culture are centrifuged and resuspended to a cell density of about 1 to 3 × 10^7/ml in 5% FCS–DMM. Animals are inoculated subcutaneously in the center of the back, not too near the neck, and high up in each flank near the spine. Mice are given 0.2 ml of cell suspension in each site and rats about twice as much, using a somewhat higher cell density. Freshly excised tumors can be used for transplanting in other animals. Tumors of a good size are sliced (discarding necrotic parts) in a petri dish containing 10 ml of Earle's balanced salt solution. A cell suspension is prepared with a loose-fitting homogenizer. The preparation can be used to inoculate about 10 other animals.

Ascitic Tumors. Before the induction of tumors mice should be inoculated intraperitoneally with 0.5 ml of pristane (tetramethylpentadecane). After 1–9 weeks about 10^7 cells are suspended in 0.5 ml of medium and injected intraperitoneally. As soon as ascitic fluid accumulates (usually about 10 days after inoculation), it is removed by "tapping" the mouse. For this a hypodermic needle (size 19, 1-inch or 20 G 1½ inch 40/9) is inserted in the abdominal area close to the surface. The liquid that drips off is collected in a suitable container. This first "tap" does not usually contain a high concentration of antibody. Further tapping should be carried out every 1–3 days. It is possible to repeat the operation perhaps 10 times without sacrificing the animal.

Control of Production

It is important to monitor the concentration of the monoclonal antibody in the serum/ascites at every tumor passage. It is not uncommon to find that on continuous passage tumors lose the capacity to produce the antibody. This is most probably due to negative variants with increased malignancy overgrowing the original positive cells. The monitoring can be done by determining the antibody titer using the most convenient assay. Alternatively, direct determination of myeloma protein concentration can be made by conventional methods. Electrophoresis on cellulose acetate strips, as routinely performed for blood samples in hospital laboratories, gives a very fast visual estimation of the concentration as well as of the mobility characteristics of the monoclonal antibody. This is a reassuring chemical check. Accidental mix-ups may not be detectable by specific antibody tests if the products are directed against a common target.

If the production of antibody declines on continuous passage, new tumors should be induced from frozen stock. If the production goes negative after only a few passages, it may be necessary to prepare a more stable clone, using the tumor cells for recloning.

C. Storage

Generally speaking monoclonal antibody can be stored as conventional antisera. Sterile samples can usually be stored for reasonable periods at 4°. Addition of preservatives such as NaN_3 at 0.1% is common. For very long periods it is probably better to store at −20°, but freezing and thawing should be avoided whenever possible, especially when dealing with IgM antibodies.

There is a critical difference between monoclonal and conventional antibodies as regards stability. Conventional antisera contain many different monoclonal antibodies, each with different stability. If only some are sensitive to a particular treatment, the activity of the preparation may not be seriously impaired by that treatment, even when done repeatedly. For instance, one out of two monoclonal antibodies to antigen X may be totally destroyed during freeze-drying, but the activity of their mixture will only decrease to 50% of the original. It is advisable to test the stability of a given monoclonal antibody to any particular treatment before committing a large batch.

XIII. Unusual Properties of Monoclonal Antibodies

When compared with ordinary antisera, monoclonal antibodies are likely to display unusual serological features. The most obvious differences arise from synergistic efects. For instance, unless the antigen contains multiple identical subunits, the monoclonal antibodies are unlikely to give precipitating reactions because no three-dimensional lattices are likely to be produced.[4]

Cytotoxicity reactions are affected not only by the class of the monoclonal antibody, but also by the local distribution of the determinants on the cell surface. This local concentration can be increased dramatically by multiple antibodies recognizing the same antigen. For instance, in a case of two different monoclonal antibodies recognizing histocompatibility antigens, neither alone is cytotoxic, but the mixture of the two is strongly so. Although these types of synergistic effects can be very confusing, they can also become very useful tools—for example, to reveal cells secreting a "nonlytic" antibody, using red cells pretreated with a previously isolated monoclonal antibody.[24]

Cooperative effects are also likely to be among the reasons why monoclonal antibodies are often less good agglutinators than the conventional (polyclonal) antisera.[22] But other facts are likely to complicate the problem. For instance, the indirect hemagglutination by a monoclonal anti-IgG was found to be very different when sheep red cells were coated with two different monoclonal anti-sheep red cells. It was *negative* when the cells were coated with Sp2 (a monoclonal antibody recognizing a *high*

density determinant) and *positive* when coating was with Sp3 (recognizing a *low* density determinant).[6] The precise reasons for the effect may involve structural features, but also the precise ratios of molecular species become critical because the reagents are monoclonal. Complex mixtures are to a certain extent self-correcting, because different antibody species present at different concentrations can act independently of each other. The use of preparations containing mixed molecules secreted by HLGK or HLK clones (Fig. 2) introduces further complications.

Precipitation analysis of labeled monoclonal antibodies mixed with polyvalent antisera is a method that is likely to be used extensively. It was observed that under these conditions the labeled monoclonal antibodies were able to diffuse through the precipitin lines to which they bind and coprecipitate with another line. This is contrary to the old assumption that precipitin lines act as diffusion barriers. One monoclonal antibody in excess may not be able to dissolve the precipitate and diffuse through it to bind to other precipitin lines containing the same determinant on a different molecular species.[22]

The fine specificity of monoclonal antibodies is a great asset but should be used with caution. Negative results with a monoclonal antibody do not prove absence of the antigen itself. Changes in the environment of the antigenic determinant, or of the way the antigen is presented, could alter results. On the other hand, reaction with a monoclonal antibody could, at least in theory, occur through recognition of more than a single antigenic structure. More commonly the same antigenic determinants could be expressed in different molecular species—e.g., carbohydrate moieties or structural features in evolutionarily related proteins.

[2] Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections

By JUDITH L. VAITUKAITIS

A wide variety of immunization techniques has been used to generate specific antisera in laboratory animals. Those techniques incorporate a variety of injection routes, vehicles, and frequencies of injection into appropriate laboratory animals. Moreover, the concentrations of immunogen have ranged from gram to milligram concentrations. With the advent of more sophisticated isolation techniques, as well as the capacity readily to synthesize polypeptides, successful immunization with small amounts of immunogen has become imperative. Consequently, an approach to

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[1] J. L. Vaitukaitis, J. B. R
33, 988 (1971).
[2] J. L. Vaitukaitis and G.
[3] H. N. Eisen and G. W.

General Comments

It was not the purpose of this review to present a comprehensive survey of hapten–protein conjugates, but rather to provide sufficient information to guide the researcher in the design of his or her particular experiments. On the other hand, the most practical approaches to the preparation of hapten–protein conjugates were cited.

Many of the methods used to prepare immunogenic conjugates have also been used to link drugs to carrier molecules (including antibodies) in order to "target" cytotoxic drugs. Two reviews that are useful in that they describe many of the methods used to make the carrier–drug conjugates are those by Trouet[131] and by Ghose.[132] The information in these reviews should be useful to immunologists as well.

[131] A. Trouet, *Eur. J. Cancer* **14**, 105 (1978).
[132] T. Ghose, *J. Natl. Cancer Inst.* **61**, 657 (1978).

[5] Production of Reagent Antibodies

By B. A. L. Hurn and Shireen M. Chantler

Immunization

The explosion of interest in immunoassay procedures during the last two decades has resulted in an enormous volume of literature describing, for the most part, satisfactory results of immunization. During the same period, knowledge of the underlying mechanisms of the immune response has advanced greatly from an original state of almost total ignorance. Perhaps unfortunately, those who have pursued basic understanding have seldom been much concerned with the practical problems of making useful reagent antibodies. As a result, with few exceptions the literature of immunization does no more than describe successful procedures, and the variety of these is legion. In the usual way of things, abortive attempts are seldom mentioned, let alone described, yet anyone with practical experience who has also discussed the matter with colleagues will be well aware that all the successful methods have also, at other times or in other places, singularly failed to give the desired results. Not surprisingly, the failure rate is higher when making antisera for more demanding test systems, such as radioimmunoassay, than for immunoprecipitin methods, for instance. Much of the uncertainty over the outcome of immunization may be ascribed to variations in individual animal response; however, when an

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experimental comparison of different procedures is made in such a way as to overcome the effect of individual animal variation, the results may well be inconclusive or irreproducible despite the considerable effort involved.[1]

Regrettably, then, it must be said that information concerning methods of immunizing laboratory animals is almost entirely anecdotal. The available evidence strongly suggests that there are influences as yet unrecognized that may be as important to success as any of the factors already known. Nevertheless, while acknowledging the significance of art, green fingers, or even plain luck, it is worth considering the known factors briefly so as to provide some evidence in support of the methods of immunization recommended later; they are related to the immunogen, the adjuvant, the choice of animal, the route of injection, and the dosage schedule.

The Immunogen

Particulate (cellular) materials, such as heterologous erythrocytes or bacteria, are usually intensely immunogenic, producing a rapid response when administered without adjuvant of any sort. The major problem likely to be encountered is lack of the desired specificity in the resultant antiserum, since the particles have a complex antigenic structure much of which may be shared with other more or less closely related cell types. Short immunization courses are usually adequate but often give rise to a high proportion of IgM antibody, which may be very satisfactory in agglutination techniques but tends to be less stable during storage than IgG.

Most antigens of interest to immunoassayists are soluble materials that vary greatly in their immunogenicity dependent on their chemical structure and molecular size. Since soluble substances are readily cleared from the circulation, either by some metabolic pathway or by excretion through routes that largely bypass lymph nodes, spleen, and other reservoirs of immunopotent cells, they rarely stimulate the production of effective reagent antibodies unless administered with some sort of adjuvant, as described below. Even then, they vary widely in immunogenicity.

Proteins and the larger polypeptides of molecular weight greater than about 5000 will readily stimulate a potent immune response. Many may exist in dimer or polymer form, either naturally or as a result of minor denaturation during purification, and this may increase their immunogenicity (major denaturation may be associated with loss of native antigenic characteristics, however, and should be avoided). The smaller the peptide

[1] S. Lader, B. A. L. Hurn, and G. Court, *in* "Radioimmunoassay and Related Procedures in Medicine," p. 31. International Atomic Energy Agency, Vienna, 1974.

within the molecular weight range of 5000–1000, the more difficult it seems to be to make avid antisera, although the correlation is much less than perfect. In this size range, closely related (or even identical) peptides are found in all the usual species of laboratory animal, so the element of "foreigness" of the antigen is lost. Many small peptides may lack the clearly defined tertiary structure that is presumably necessary for a substance to be recognized as a unique antigen. Finally, degradation of these substances in the tissues and circulation, by specific enzymes and by nonspecific proteases, may well be so brisk as to prevent effective contact with immunopotent cells.

With the exception of some of the larger polysaccharide molecules, no substances other than the proteins and larger polypeptides are effective immunogens in themselves. Nevertheless, antisera of high avidity and specificity can be raised to steroids, glycosides, oligopeptides, and the like if they are first chemically bonded to a large carrier molecule, preferably a protein that is in itself immunogenic in the species under immunization. Current immunological theory suggests that the initial stages of immunization require cooperation between T and B lymphocytes, the T lymphocytes first binding with a recognizably "foreign" substance and then presenting the bound antigen to B lymphocytes bearing suitable receptors. This cooperation is impossible if the antigen is too small to be shared between T and B cells, but a complex of the antigen with a suitable carrier becomes fully effective. Small, nonimmunogenic antigens of this type are known as haptens and, in the form of drugs, steroid hormones and small peptides, have been of great interest to immunoassayists during the last decade. The method of coupling carrier and hapten should be carefully chosen so as to avoid unwanted structural alteration of the latter and so that the linkage does not involve the immunochemically distinctive part of the hapten molecule. Antibodies produced in response to immunization with conjugated haptens generally "recognize" that part of the hapten farthest from the point of linkage, which thus determines their specificity. Highly substituted carriers are usually most effective, and molar ratios of 15–30:1 (hapten:carrier) are desirable, when possible. For best results the carrier should be a protein foreign to the immunized species—thyroglobulin and keyhole limpet hemocyanin are used quite widely, but bovine (or other) serum albumin is fully effective and more easily available. The subject has recently been well reviewed in relation to steroid conjugates by Pratt.[2]

The purity of the immunogen is of controversial importance. For synthetic substances, however, no argument exists—the likelihood of closely related substances (such as "error peptides") being present in impure preparations, subsequently leading to the most objectionable variety of nonspecific antibody, means that maximum possible purity is essential. For particulate antigens, especially bacteria, there is also no reason for lack of purity, but the needs in respect to soluble substances extracted from natural sources are somewhat different. There is no doubt that relatively crude preparations are highly immunogenic, often more so than purer materials, so that many workers have thought of the impurities as having some adjuvant-like activity. The probability, however, is that greater purification has led to concomitant subtle chemical changes (such as deamidation) so that the immunogen stimulates antibodies that fail, to a greater or lesser extent, to "see" the native antigen. Despite this, a high degree of purification of immunogen must sometimes be sought in order to eliminate certain types of cross-reactivity in antisera. At the other extreme, gross impurity should be avoided, even when the cross-reactions are unimportant, because antigenic competition may then prevent formation of any specific antibody. In practical terms, about 10% purity is the minimum required to make a significant specific antibody response reasonably likely.

[2] J. J. Pratt, *Clin. Chem.* **24**, 1869 (1978).

The Adjuvant

A wide variety of substances are known to have the property of potentiating the humoral antibody response to injected immunogen. Among them are inorganic adsorbents, such as aluminum hydroxide gel; mineral oils, such as liquid paraffin; and bacterial cell wall components. The diversity of materials having adjuvant properties, which has been the subject of a recent review by Whitehouse,[3] makes it difficult to identify a simple mechanism of action. Three major effects are involved, albeit to different degrees for each adjuvant type. First, the release of immunogen from the site of injection is slowed, either by adsorption to solid particles or by incorporation into an oily emulsion. This leads to a "sustained release" from a depot at the injection site, where labile immunogens are also protected from breakdown by tissue enzymes. A secondary benefit is that any direct toxic effects of the immunogen on the recipient will be minimized. Second, adjuvants have a stimulatory effect on reticuloendothelial cells, attracting a local infiltration of the injection area by mononuclear cells and stimulating phagocytosis by macrophages presumably by presenting soluble immunogen in a particulate or partially aggregated form. Adjuvant-treated macrophages with antigen inoculated into histocompatible recipients give rise to a higher antibody response than transfer

[3] M. W. Whitehouse, *in* "Immunochemistry: An Advanced Textbook" (L. E. Glynn and M. W. Steward, eds.), p. 571. Wiley, New York, 1977.

of macrophages containing antigen alone.[4] Third, it has been shown that adjuvants induce an increased circulation of lymphocytes through lymphoid tissues in the drainage area,[5] macrophages being important for the initiation of these lymphocyte traffic changes.[6] The increased flow of cells in regional lymph nodes is likely to allow greater contact between antigen and antigen-reactive cells, thus facilitating increased antibody production. The local granulomatous lesions formed at the sites of injection may also serve as foci of antibody production.

The most important advance in adjuvant technology arose from the observation of Dienes and Schoenheit[7] that antigen injected into tuberculous granulomata stimulated higher antibody titers than did antigen injected at nontuberculous sites. These findings led Freund and co-workers to develop a series of adjuvants containing mycobacteria, mineral oil, and emulsifier,[8] which to this day remain the most potent tools available to the aspiring immunologist. The mixture most widely used in the preparation of reagent antibodies contains 9 parts of mineral oil to 1 part of detergent. The detergent (usually Arlacel A) contains a high level of both hydrophilic and lipophilic groups, thus facilitating dispersion of the oily and aqueous (immunogen) phases and allowing the formation of a stable emulsion. The simple oil–detergent mixtures are termed "incomplete" Freund's adjuvant; incorporation of heat-killed *Mycobacterium tuberculosis* or *M. butyricum* (0.5 mg/ml) into the oily mixture yields "complete" Freund's adjuvant. The latter is the more effective, probably as a result of greater stimulation of the local cellular response, and must now be regarded as an essential aid to the production of reagent antibodies against soluble immunogens.

Preparation of Freund's Emulsions. For maximum efficiency, it is necessary to obtain a stable, water-in-oil emulsion. Several ways of preparing such emulsions have been described, but there is no doubt that the simplest and most efficient, at least for the relatively small volumes that most people require, is the double-hub connector method described here. It may occasionally be difficult to persuade the phases to combine as water in oil rather than oil in water or mixed emulsions. Cooling the separate phases before mixing may help, but an infallible way of overcoming the problem is to use 2–4 volumes of oily adjuvant to 1 volume of aqueous

[4] E. R. Unanue, B. A. Askonas, and A. C. Allison, *J. Immunol.* **103,** 71 (1969).
[5] P. Frost and E. M. Lance, *in* "Immunopotentiation," CIBA Found. Symp. 18 (New series), p. 29. Excerpta Medica, Amsterdam, 1973.
[6] P. Frost and E. M. Lance, *Immunology* **26,** 175 (1974).
[7] L. Dienes and E. W. Schoenheit, *J. Immunol.* **19,** 41 (1930).
[8] J. Freund and K. McDermott, *Proc. Soc. Exp. Biol. Med.* **49,** 548 (1942).

immunogen. Experience has shown these oil-rich emulsions to be at least as effective (probably more effective) than the 1 : 1 ratio usually recommended. A subsidiary advantage is that they flow more easily, so both mixing and injection are less of a chore. If it is essential to use a 1 : 1 ratio (because of volume restrictions when the immunogen solution is very dilute, for instance), the formation of water in oil emulsions can be reliably achieved by adding the aqueous phase in three increments, mixing after each.

The necessary apparatus consists of the double-hub connector and two syringes, each large enough to contain the total emulsion volume without overfilling. The largest practicable volume that can be handled by someone with averagely large, reasonably powerful hands is 14–16 ml, using two 20-ml syringes. The best type of syringe for the purpose is an all-glass, center-hub pattern; metal-and-glass types tend to leak at the piston, and the common plastic syringes become very stiff while making the emulsion. Plastic syringes are reasonably satisfactory in the smaller sizes, however, since less force is needed for small volumes.

If Freund's complete adjuvant is required, shake it very thoroughly to resuspend the bacterial cells immediately before use. Pour out sufficient of the adjuvant into a small beaker (to avoid contaminating the remainder) and draw the required volume up into one of the syringes. Attach the double-hub connector, and carefully expel all air until the oil rises up into the farther end of the connector. Draw the aqueous immunogen into the other syringe, remove the needle, and again expel all air until the syringe hub is full of liquid, then connect it to the open end of the connector: any air left in the apparatus will be trapped in the emulsion and, because of its compressibility, will make injections more difficult. At this stage make sure that both syringes are firmly inserted into the connector, but be careful from now on not to place any bending stress on the rather unwieldly apparatus, especially if the syringes have glass hubs. A little oil will almost certainly be squeezed out of the connector (the whole process is somewhat messy) and it may be as well to wipe the apparatus and fingers with a tissue before proceeding.

To form the emulsion, begin by squirting the aqueous phase into the oil as vigorously as possible, then continue squirting the total contents to-and-fro from one syringe to the other a minimum of 10 times each way (20 times is better, if your thumbs can stand it). To avoid bending stress at the connections, practise deliberate relaxation of the "receiving" hand so that the filling syringe just rests on the palm as the other hand is grasping and pressing on the plunger. Especially as the hands tire it is tempting to let the receiving hand try to help the other, but this inevitably places strain on the syringe hubs. A fracture of a hub (or sudden falling apart of a

:arelessly made connection) causes an explosive shower of emulsion to :ontaminate everything with a radius of several feet (including the opera- or's face) and is sufficiently unpleasant to encourage more care thereafter.

If the aqueous phase is to be added in several aliquots in order to promote the formation of water-in-oil emulsions at the 1:1 ratio, it will be necessary to break one of the connections each time more of the water phase is needed. About five each-way strokes of the syringe will be sufficient for the intermediate mixing, after which the next aliquot of immunogen is taken up into the empty syringe, the connection is made again and, as before, mixing begins by squirting the water into the oily emulsion. Repeated disconnection and reconnection make it all the more difficult to exclude air and prevent messy oil from leaking out: it is better to use a larger oil to water ratio and avoid the problem altogether whenever possible.

In the authors' experience, the above method will lead infallibly to the proper type of emulsion, and testing is therefore unnecessary. For those who wish to confirm success, however, the simplest way is to take a beaker half full of water and drop two small, separate drops of emulsion onto the water surface. The first drop always spreads somewhat, but the second will remain a discrete, white globule with no spreading at all if the emulsion is, indeed, water in oil. If the second drop disintegrates into bits and pieces that spread around over the surface of the water, the emulsion was oil in water, at least in part, and should be prepared afresh. Read the above instructions again first, though.

After use, plastic syringes should be thrown away, but other apparatus must be washed up. The connector can first be pushed into a piece of rubber tubing connected to a hot tap and flushed through for a few minutes. Syringes should be cleaned with washing-up detergent, then soaked, together with the connector, in a decontaminating detergent (such as Decon 90) for a day or two before rinsing and drying. Residues from emulsions are probably difficult to remove completely, and the syringes should never again be used for any purpose other than preparing such materials.

The Choice of Animal

There are few instances in which categorical evidence has shown one species of common laboratory animal to give consistently better responses than another to any particular immunogen. Some fairly well known exceptions are the superiority of guinea pigs for production of anti-insulin sera (presumably because the endogenous hormone in this species is most unlike the other mammalian insulins) and of horses for preparation of antisera for immunoelectrophoresis. The latter preference is due to the

solubility of horse antibody immune precipitates in excess antibody (all immune precipitates are soluble in antigen excess) yielding unusually narrow, clear-cut precipitin arcs. Apart from these examples, however, the literature abounds with indications of the personal preferences of the authors for which the evidence is apocryphal and often contradictory.

In most instances the choice of species may reasonably be made on the basis of what is available and the volume of antiserum required—the larger the animal, the bigger the yield. It will be understood that it is usually sensible to immunize a species that is "foreign" to the antigen in question. If homologous immunogens are used, it should be for a valid reason (production of tissue typing sera, for instance, relies on antibodies produced in the same species as the donor). Homologous immunization, when it produces a result at all, will yield antibodies that recognize fine, interindividual differences in the antigen; by contrast, immunization of a foreign species readily yields much more abundant antibody but any reactivity with the structurally minor, idiotypic variants of the antigen is almost always lost in the reactivity against the gross, interspecies difference.

A well provided laboratory may have access to guinea pigs, rabbits, sheep or goats, donkeys, and horses. There is little doubt that rabbits should be the first choice for most purposes unless very large amounts of serum are needed. Rabbits are cheap, easy to care for, robust in the face of quite intensive immunization, and easy to bleed. The other species may best be held in reserve in case of a failure with rabbits. Another reserve species that may be available is the chicken—again quite easy to handle, but producing antibodies that behave differently from those of mammalian species[9] and hence best avoided if possible.

Whichever species is chosen, it pays to immunize several individuals (which is a good reason for avoiding the larger, more expensive species to begin with). Individual variation in response is often very striking, especially to the more "difficult" immunogens, and groups of at least four or five animals should be started if any difficulty whatsoever is to be expected in preparing satisfactory antisera. Nonproductive animals can be disposed of once it is clear that they will not improve (this may not be for several months with some immunogens) whereas the better responders can be kept under immunization for a year or more and bled repeatedly. Obviously, such a course cannot be followed where the early antibody is desired, for instance in the production of hemolytic serum with minimal hemagglutinating activity for use in complement fixation tests.

[9] A. A. Benedict, *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. 1, p. 229. Academic Press, New York, 1967.

Immune responsiveness to certain antigens has been shown to be genetically determined.[10] The importance of this in the context of antigens of general interest is not known, but it would seem to be desirable to use random-bred animals whenever possible, to give the best chance of a good response in one or more, unless previous experience has already shown that a particular inbred strain responds well to the immunogen in question. Whatever animals be chosen, they should be kept clean, healthy, and well fed if they are to perform well as antibody factories. The subject of animal husbandry is dealt with in a number of works (see, e.g., Short and Woodnott[11] and Chase[12]) but is, perhaps, of no direct interest to the readers of this chapter.

The Route of Injection

For soluble immunogens, it is generally believed that the efficiency of stimulation of the immune response is related to the site of inoculation. A probable series, in order of increasing effect, is intravenous < intramuscular < subcutaneous < intraperitoneal < intradermal < intraarticular < intranodal. The principal reasons for the differences in efficiency are the speed with which antigen is lost from the site of injection and the likelihood of it passing through the lymph nodes or other centers of immunological activity on the way. These considerations, however, are radically affected by the use of adjuvants, especially oily adjuvants, which may stimulate a brisk local cellular reaction and release antigen over a period of several weeks or even months.

Using oily adjuvants, then, the injection site can be chosen principally with a view to minimizing discomfort to the animal. Generally this means intramuscular injections in rabbits and larger animals or subcutaneous injections in guinea pigs; note that water in oil emulsions must never be given intravenously because of the virtual certainty of fatal fat embolism. Subcutaneous or intradermal injection of Freund's emulsions almost invariably leads to ulceration, but provided the sites are well chosen (see below) rabbits and guinea pigs show no sign of distress or loss of condition. Some authors (see Herbert[13]) have suggested that Freund's emulsions should not be injected subcutaneously since ulceration may lead to

[10] I. Green, W. E. Paul, and B. Benacerraf, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1095 (1969).
[11] D. J. Short and D. P. Woodnott, eds., "The I.A.T. Manual of Laboratory Animal Practice and Techniques," 2nd ed. Crosby Lockwood, London, 1969.
[12] M. W. Chase, *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. 1, p. 254. Academic Press, New York, 1967.
[13] W. J. Herbert, *In* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 2nd ed., App. 2. Blackwell, Oxford, 1973.

loss of the depot: in the experience of the present authors this has never given rise to difficulty. Occasionally deep abscesses form after intramuscular injection and lead to loss of condition. The abscesses are frequently "sterile" and, in our experience, have usually been related to overzealous attempts to improve on sterile injection techniques (cleaning the skin over the injection area, for instance) rather than to the use of unsterile immunogens.

Difficulties in preparing antisera against some of the antigens of interest in radioimmunoassay have led people to try a wide variety of methods of immunization. Most of these variations have been irrational (which does not mean to say they have not worked on occasion), but two deserve special mention. By injection of immunogen (angiotensin I, adsorbed on carbon black and emulsified in Freund's adjuvant) directly into rabbit lymph nodes and spleen, Boyd and Peart[14] obtained improved results that they believed to be due to more direct stimulation of the immune system. A subsequent comparative trial gave rather equivocal results,[15] however, and the method was too difficult to be widely used. Injection into the Peyer's patches (lymphoid patches in the intestinal wall, quite easily visible in the rabbit) is technically much simpler but has proved no more successful in the authors' hands.

Much simpler than the intranodal method, and now quite widely used, is the method of multiple intradermal inoculation introduced by Vaitukaitis *et al.*[16] The immunogen is introduced at 40 or more sites spread widely over the body surface. Antibody response to this primary immunization is much greater than to a first injection given in the usual way, and no more than one booster injection is usually required. Comparison with the usual intramuscular injection schedule[1] showed no great difference in efficiency, although the multiple intradermal technique (with only one booster) required rather less immunogen and yielded effective antisera in a shorter period of time.

The Dosage of Immunogen and Timing of Injections

Although an animal may be made "tolerant" to soluble antigens given in too low or too high a dose under certain circumstances, the use of a potent adjuvant makes such an outcome extremely unlikely. Nevertheless, the observation that too high a dose can lead to antiserum of rela-

[14] G. W. Boyd and W. S. Peart, *Lancet* **2**, 129 (1968).
[15] B. A. L. Hurn and J. Landon, *in* "Radioimmunoassay Methods" (K. E. Kirkham and W. M. Hunter, eds.), p. 121. Churchill Livingstone, Edinburgh, 1971.
[16] J. Vaitukaitis, J. B. Robbins, E. Nieschlag, and G. T. Ross, *J. Clin. Endocrinol.* **33**, 988 (1971).

tively low avidity,[17,18] presumably owing to stimulation of lymphocytes bearing low-affinity receptors, may certainly be relevant even when using Freund's adjuvant. Since most sensitive immunoassay techniques of current interest rely on antibody of the highest possible avidity, it is evidently desirable (and economical of immunogen) to use the lowest dose that will be fully effective. This dose is very much smaller than most of the published literature recognizes, and a suitable priming (first) inoculation for rabbits or guinea pigs will generally be of the order of 100 μg. A range of 50–1000 μg should cover all needs, depending on the purity and immunogenicity of the material in question (but it is sensible to start at the lower end, since an animal showing lack of response after a sufficiently long trial can then be given a larger dose, whereas an animal producing poor antiserum after high dosage is beyond hope of salvage). The dosage required for larger animals does not increase in proportion to body weight: 0.25–5 mg is satisfactory for sheep and 0.5–10 mg for donkeys. For conjugated haptens, incidentally, these figures refer to total conjugate weight.

Booster injections are always needed to obtain antisera of the highest titer and avidity. Practical experience suggests that good results will be obtained using a booster dose about half the size of an effective priming dose, given by the same route (not necessarily at the same site) and using Freund's complete adjuvant on each occasion. It is recognized that these recommendations are somewhat at variance both with immunological theory (which would suggest a progressive increase in dose) and with the advice of other authors to avoid repeated use of Freund's complete adjuvant, especially subcutaneously, because of abscess formation and hypersensitivity reactions. There is some documented evidence in support of the suggested reduction in dose,[1] but the repeated use of complete Freund's adjuvant is a recommendation that stems only from satisfactory, albeit uncontrolled, experience.

The repeated booster doses that are usually required for the best antiserum should not be given too frequently. It has been shown[19] that no further rise in titer results from a second injection given before the response to the first is reaching its peak. At least 4 weeks should pass between injections of Freund's emulsions. After the first booster, or sometimes after the second, antibody response may be quite prolonged and many people believe that a rest of 3–6 months is desirable before the next injection if antiserum of the highest avidity is required; the evidence in favor of this approach is not strong,[1] but in general terms there is little doubt that patience is desirable when making reagent antibodies. It is not unusual to read descriptions of immunization schedules involving weekly injections of quite large amounts of immunogen in Freund's emulsion; published accounts, not surprisingly, tend to report a successful outcome, but the approach is not to be recommended.

[17] G. W. Siskind and B. Benacerraf, *Adv. Immunol.* **10**, 1 (1969).
[18] E. J. Greene and J. G. Tew, *Cell. Immunol.* **26**, 1 (1976).
[19] W. J. Herbert, *Immunology* **14**, 301 (1968).

Many published immunization procedures terminate with one or more intravenous injections of soluble immunogen given without adjuvant after a course of intramuscular Freund's emulsions. In the authors' experience, this produces a less satisfactory response (about half the final titer of avid antibody) than can be obtained with a final injection of intramuscular emulsion.

By contrast with the above, particulate immunogens are normally administered intravenously, frequently (perhaps every other day), in increasing doses and for short periods of time. These materials are usually highly immunogenic, partly because the normal mechanism for their removal brings them into close contact with the immune system and partly because many of them (notably bacterial cells) are antigenically very "foreign" to the immunized animal. Antibody production is rapid, and the early IgM response is excellent for agglutination tests. Initial doses of immunogen are extremely variable, owing to the variable toxicity of the substances concerned (especially bacteria containing endotoxins), and for many of the antigens hypersensitivity reactions to later doses may prove rapidly lethal. Subcutaneous injection, with relatively slow absorption, may ameliorate undesirable acute reactions.

Although short immunization courses for particulate antigens are the rule, usually in the belief that antisera will become less specific as immunization proceeds, this is not necessarily the case. Prolonged immunization may result in more stable IgG antibody of higher titer and, because of repeated bleeding over a period of time, in much greater yield.

Practical Immunization Schedules

Animals often remain under immunization for many months, even years. You may not be personally responsible for their care during this time, but in your own interests you must ensure that either the individual animals or their cages are properly labeled in a manner compatible with your own records at the time of the first injection so that the individual animals can be identified with certainty thereafter. If the cages alone are labeled, you would also be advised to ensure that the method of animal handling, especially during cage cleaning, is such as to prevent animals being moved accidentally from one cage to another.

Rabbits

Four or more healthy, young adult rabbits should be treated with each immunogen.

Soluble Immunogens

Either the intramuscular or the multiple intradermal route may be recommended. As examples of representative immunogens for which high-avidity antisera are required, consider a crude preparation of human chorionic gonadotropin (hCG) and the beta subunit of hCG (β-hCG). The former, at a characteristic potency of 1500–3000 IU/mg, is about 20% pure whereas the latter is of necessity highly purified and in short supply. Appropriate doses for primary immunization are 1 mg and 100 μg, respectively. Booster doses should be half these amounts.

Dissolve the immunogen in isotonic saline (other immunogens may require slight acidity, alkalinity, or other special condition) to a volume of 0.5 ml per rabbit for the primary injection or 0.25 ml for boosters (i.e., the same concentration for both injections). Emulsify the solution with three volumes of Freund's complete adjuvant, using a double-hub connector and two syringes as described above. The total volume of emulsion will then be 2 ml per rabbit for the primary inoculation or 1 ml for a booster. Use the emulsion within an hour of preparation.

Intramuscular Schedule. Do not shave the animals or attempt to prepare the skin in any way prior to injection. A fairly stout needle of medium length (21 gauge × 1 inch) is convenient and need not be changed between animals unless it becomes blunted for any reason. Injections are given into thigh and/or upper foreleg muscle, where thickest, and the hair can be parted by gently blowing down on to the selected site immediately before injection.

For the primary injection, give 0.5 ml of emulsion intramuscularly into each of the four limbs of each animal. Now go away and think about other things for *at least* 4 weeks, or 6 weeks if possible.

For booster injections, give 0.5 ml of emulsion intramuscularly either into each hind limb or into each fore limb, alternately. Bleeds (20–40 ml) may be taken for testing on two occasions between 7 and 10 days after each booster and similarly every 3–4 weeks thereafter if the antiserum is satisfactory. Further boosters may be given at *minimum* intervals of 4 weeks (but preferably not within 2 weeks of a bleed) although it may pay to rest the animal for 3–4 months after the second or third booster.

Animals that fail to show a reasonable response after two or three boosters should be disposed of. This decision must be related to the level of response expected for the particular immunogen used—some animals

may take several months to respond to "difficult" immunogens, and early responders are not necessarily the best in the end.

Multiple Intradermal Method. Shave the hair on the back and on the proximal parts of all four limbs of each rabbit. As a guide to spacing the injections, draw six transverse lines across the shaved area of the back, using a felt-tip marker. The injections should be made with a tuberculin syringe and a fine needle (the syringe holds only enough for one animal but may be loaded repeatedly from the syringe in which the emulsion has been prepared, via the double-hub connector).

Make 24 intradermal injections each of 0.05 ml, spaced evenly over the back. Distribute the remainder of the emulsion (about 0.8 ml, or sixteen 0.05 ml injections) over the inner and outer aspects of each upper limb, in the shaved areas. Satisfactory intradermal injections are easily recognized by a characteristic, localized bleb; this is easy to achieve on the back of the animal, where the dermis is quite thick and tough, but very difficult on the limbs, where the skin is much more delicate. Try, but do not be unduly discouraged if you fail.

Within a few days of the injections the rabbit will present a horrifying sight, covered as it will be with forty, half-inch ulcers. In the authors' experience, the animals are happily unaware of the aesthetics of the situation and continue to thrive without any specific treatment. Some users of the technique have found otherwise, for no known reason. In the interest of animal welfare, if you find your rabbits are greatly upset by this procedure then please revert to the intramuscular procedure, which can be just as effective.

After the multiple injections the animals should be left for *at least* 10 weeks before boosting. Antibody levels rise to relatively high titers during this time, however, and it is certainly worth taking a large bleed for testing after 8–10 weeks. All booster injections are given by the intramuscular route, and the method of treatment from the tenth week onward is thus exactly the same as for the previous schedule.

Particulate Immunogens

These antigens are commonly administered by frequent, intravenous injection without adjuvant. Results are obtained quickly, the antisera often containing a high proportion of IgM immunoglobulin. There is a risk both of direct toxicity early in immunization and of severe hypersensitivity reactions as a result of later injections. With some at least of the antigens in question, equally satisfactory results can be obtained by intramuscular injection of Freund's emulsions—immunization is slower but less risky.

Production of antisera to *Escherichia coli*, for use as specific typing reagents, furnishes an example of a typical intravenous schedule. Good bacteriological technique and the selection of an appropriate colonial form of the organism is essential to the specificity and reactivity of the antiserum (this is obviously analogous to the purification of a soluble immunogen). Living organisms are required for expression of the important K antigens in this species, but live coli will kill a high proportion of unprotected animals and the early injections are therefore made with heat-killed suspensions. Antisera to most other microbial species can be prepared against killed suspensions throughout. The following schedule should be followed (all suspensions being prepared to an opacity of Brown's tube 4, and all injections given intravenously).

Day 1: 0.25 ml of killed suspension
Day 3: 1.0 ml of killed suspension
Day 5: 3.0 ml of killed suspension
Day 9: 0.5 ml of freshly prepared living suspension
Day 12: 1.0 ml of freshly prepared living suspension
Day 16: 3.0 ml of freshly prepared living suspension
Day 22: test bleed for titer
Either
Day 23 Bleed out if titer is satisfactory
Or
Continue weekly injections as for day 16 with test bleeds 5–7 days later, until satisfactory titers are obtained.

Guinea Pigs

Each animal will yield only 3–5 ml of serum by cardiac puncture or 15–25 ml when bled out. For this reason guinea pigs are best reserved for use when only small quantities of antiserum are required (particularly in radioimmunoassay and similar immunoassays) or when other animals are known not to respond well to the immunogen in question (insulin is such a substance, and, in our experience, parathyroid hormone is another). Groups of up to 10 guinea pigs may conveniently be kept in a single large cage, individuals being identified by natural markings or applied pigments (the latter need to be renewed rather frequently).

Soluble immunogens should be administered as Freund's emulsions, injected subcutaneously into the abdominal wall just on either side of the midline. The injection sites will usually ulcerate after a week or so, but the animals are apparently free from discomfort, thrive, and make good antibodies.

Prepare the inoculum by emulsifying 1 volume of aqueous immunogen

in 2–3 volumes of Freund's complete adjuvant in the usual way, to give a total volume of 0.5 ml per animal. Injections should be given at intervals of not less than 4 weeks although longer rests later in the course of immunization may be desirable. Because of the low yield of serum and the risk of killing the animals when bleeding by cardiac puncture, it is less practicable to bleed guinea pigs repeatedly than it is to bleed rabbits. Since guinea pigs are cheaper to buy and look after, it is probably best to immunize a relatively large number for a comparatively long period of time, then bleed them out and select the best antisera from the result. Our experience has suggested that at least four injections are desirable if this strategy is employed, and six injections may often be better. The decision depends on the purpose for which the antiserum is required and, in particular, whether the highest possible avidity is needed.

Sheep

The immunization of sheep offers the possibility of obtaining relatively large amounts of antiserum, not only because each individual bleed is larger (150–300 ml of serum, depending on the size of the animal), but also because the animals may be maintained and bled repeatedly for longer than rabbits. This can be a major advantage when antisera are to be prepared for relatively undemanding, insensitive test systems such as immunoprecipitation, when larger volumes of reagent are required but variations in quality over the course of time are unlikely to cause difficulty. The higher cost of buying and keeping a sheep makes it less attractive when the use of a "difficult" immunogen makes it necessary to immunize a large number of animals. Circumstances alter cases, of course, and an Australian laboratory might have a different view of the relative economy of sheep and rabbits.

Immunization of a sheep should proceed according to a schedule similar to that described for a rabbit. Intramuscular injections (as usual, always prepared with Freund's complete adjuvant) should be given with a 1½–2-inch needle deeply into the haunch or shoulder (preferably into all four "corners" for the first injection). As has been mentioned before, dosage is not proportional to size and for a relatively good immunogen such as human IgG an initial injection of 0.2–1 mg, followed by booster doses of half that size, should be sufficient. After the first two or three monthly injections, subsequent boosts should be given at longer intervals depending on the quality of antiserum. Bleeds may be collected on a regular schedule throughout the period of immunization, the best yields being obtained if three bleeds (of 300–600 ml, depending on the size and experience of the animal) are taken over a period of 8–10 days followed by

ɔut 3 weeks rest before the next triple bleed. A healthy animal may re-
ain productive for some considerable time, at least a year or two, but
:ibody levels will eventually decay and fail to respond to a further
oster injection, at which time the animal should be disposed of.

Collection and Storage of Immune Serum

Animals immunized with Freund's emulsions should be bled 7–10
ys after booster injections. If the blood is taken from a vein rather than
cardiac puncture, two or three bleeds can be taken on successive days,
t the animal should then be rested for 3–4 weeks before further bleed-
; or before boosting again if the original antiserum was not of satisfac-
y quality. After intravenous injection antibody levels rise and then fall
ɔre rapidly and bleeds should be collected 5–7 days after the last dose.
s often helpful to fast the animals overnight to minimize lipemia, but do
t deprive them of drinking water.

Blood should be collected in clean, dry, glass bottles and allowed to
ɔt at room temperature or at 37° until the clot has retracted; it may help
"ring" the clot with a glass rod to promote separation. The sample
ɔuld then be centrifuged and the serum be separated without undue
lay in order to avoid unnecessary hemolysis, which looks unaesthetic
hough it has no obvious deleterious effect on the antibody. When han-
ng large quantities of blood it may be easier to separate serum from the
ɔt by letting it drain through a stainless steel mesh cone supported in a
er funnel—this can even be left to drain overnight in the cold room if
: maximum possible yield is required, but in any case a final centrifuga-
n will be required to remove residual red cells.

After separation from the clot, antiserum may be stored without signif-
nt deterioration for long periods of time under a variety of conditions.[20]
:ounsel of perfection for reference or otherwise most precious reagents
ɔuld be to filter sterilize, fill out in appropriate, accurately measured,
all amounts (diluting in a suitable carrier medium if necessary), and
·n freeze-dry prior to storage at 4° or below. Experience has shown,
wever, that IgG antibodies are remarkably robust and that liquid anti-
um (even without sterilization) can be kept for many months at 4° with
% sodium azide added as an antibacterial agent. Storage at about −20°
he ordinary laboratory freezer cabinet is, in theory, likely to cause pro-
n denaturation due to the proximity of this temperature to the eutectic
sodium chloride (the complex mixture comprising serum will not be

:. E. Kirkham and W. M. Hunter, eds., *in* "Radioimmunoassay Methods," pp. 189–193.
'hurchill Livingstone, Edinburgh, 1971.

completely frozen at −20°) but, again in practice, the freezer has proved most convenient and harmless to antibody protein provided that repeated freezing and thawing is avoided. Storage at lower temperature, preferably not in unreliable mechanical refrigerators, is very satisfactory when available. Gas-phase liquid nitrogen is the ideal low-temperature storage medium, being more reliable and convenient than mechanical or CO_2 cabinets, not involving the special restrictions on storage vessels imposed by immersion storage in liquid nitrogen yet virtually guaranteeing lifetime stability of precious antisera (the investigator's lifetime, that is to say).

Storage of IgM antibodies is far more of a problem and gives very variable results. Most antisera containing IgM can be handled exactly as described above, with only gradual deterioration that would be inapparent in the relatively undemanding test systems in which this class of antibody is generally used. Some, on the other hand, prove much less stable. On occasions, this instability is associated with bacterial growth (which seldom causes much loss of IgG antibody activity although it is embarrassing and should be avoided if possible). For this reason it is strongly recommended that IgM antisera should have 0.1% sodium azide added, be sterilized by filtration at the earliest possible opportunity (before bacterial growth and release of enzymes can occur) and be handled in a cleanly fashion thereafter.

Even when collected after overnight fasting of the animal, defatted (see below), sterilized and with a bacteriostat added, serum stored at 4° will gradually become turbid and show a deposit, principally of denatured lipoprotein. This does not lead to any loss of antibody activity although it is easily mistaken for bacterial contamination and causes anxiety for that reason. The only practical disadvantage is seen when the antiserum is used in capillary precipitin reactions, when the turbidity can obscure the result unless the antiserum is first clarified by filtration.

Further Treatment of Antisera

Defatting Antiserum[21]

Antisera to be used in capillary precipitin tests must be crystal clear so that the faint ring of precipitation can be easily seen. Untreated sera become turbid on storage, due to precipitation of denatured lipoprotein; such precipitates can be removed by membrane filtration prior to use, but it is usually better to reduce the severity of the problem by extracting the bulk of the lipoprotein at the time the serum is first prepared.

[21] A. S. McFarlane, *Nature (London)* **149**, 439 (1942).

Materials

Diethyl ether, solvent grade
Solid CO_2–methylated spirit freezing bath

Procedure

1. Place the serum in a beaker and add 3 ml of ether for every 10 ml of serum.
2. Place the beaker in the freezing bath.
3. Stir the serum–ether mixture quite briskly with a glass rod until it has frozen solid. The two liquids are completely miscible in these proportions at the freezing point.
4. Allow the frozen mixture to stand in the freezing bath for another 10 min, then remove the beaker and stand it in tepid water until the frozen plug loosens.
5. As soon as possible, tip the still frozen plug into a glass filter funnel (without filter) leading into a cylindrical separating funnel. Make sure the stopcock on the latter is free running and well lubricated with a silicone grease.
6. Allow the frozen material to thaw and run into the separating funnel at room temperature, then remove the filter funnel and close the separating funnel with a rubber stopper covered in metal foil.
7. Allow the separating funnel to stand undisturbed, at 4° if possible, overnight.
8. The next day the serum–ether emulsion will have separated into a lower layer of clear serum shading gradually into an opalescent zone of residual emulsion that has a sharp interface with the uppermost, opaque, fatty layer. Collect the serum by running off the bottom and intermediate layers.
9. Remove the bulk of the residual ether by boiling off under reduced pressure, ideally with the aid of a rotary evaporator.
10. Add preservative and sterilize the serum by filtration prior to storage.

NOTE: Due care should be taken to avoid the risk of fire or explosion when handling ether.

Absorption of Nonspecific Antisera

The production of potent antiserum almost always results in a reagent with some degree of reactivity against nonspecific antigens, either because of impurities in the immunogen used or because there are "shared determinants" present in both specific and nonspecific antigens. Whatever the cause of the unwanted reactivity, it is usually necessary to remove it by absorbing the antiserum with an appropriate antigen. Although absorption may be carried out with solutions of the antigen (the immune precipitate being removed afterward by centrifugation or filtration), excess antigen or soluble complexes of antibody and antigen will inevitably remain in the absorbed antiserum. A more satisfactory method, therefore, is the use of a solid phase immunoadsorbent prepared from the appropriate antigen, which can be added in excess and easily recovered for later re-use if required. Such adsorbents may be made from antigen alone by the use of cross-linking reagents (such as glutaraldehyde for protein antigens) or can be more complex reagents prepared by chemical coupling of the antigen to a solid support such as Sepharose. The latter technique is covered in a subsequent section on the use of IgG–Sepharose immunoadsorbent, but the present example describes the preparation of a glutaraldehyde polymer of $F(ab')_2$ suitable for removal of light-chain cross-reactivity from class-specific anti-immunoglobulin sera. It should be noted that the pH optimum for efficient polymerization by this method varies considerably depending on the protein to be treated; if a polymer of whole serum is required, for instance, a pH of 4.4 will be optimal.

Preparation of $F(ab')_2$ Immunoadsorbent by Glutaraldehyde Polymerization[22]

Materials

$F(ab')_2$ prepared by pepsin digestion of IgG[23]
Phosphate buffer, 0.1 *M*, pH 7.0
Glutaraldehyde, 2% in saline
Glycine-HCl buffer, 0.1 *M*, pH 2.5
Tris-HCl, 0.1 *M*, pH 8.0
Phosphate-buffered saline, 10m*M*, pH 7.5 (PBS)

Procedure

1. Dialyze 100 mg of $F(ab')_2$ preparation (20–50 mg/ml) against phosphate buffer at 4° overnight.
2. Place the $F(ab')_2$ solution on magnetic stirrer and add 0.4 ml of glutaraldehyde solution dropwise from a Pasteur pipette.
3. Allow the gel that forms to remain at room temperature for 3 hr and then place at 4° overnight.
4. Homogenize the gel in phosphate buffer then centrifuge hard in a bench centrifuge and discard the supernatant.
5. Repeat step 4 using glycine-HCl buffer.

[22] S. Avrameas and T. Ternynck, *Immunochemistry* **6**, 53 (1969).
[23] L. H. Madsen and L. S. Rodkey, *J. Immunol. Methods* **9**, 355 (1976).

6. Repeat step 4 using Tris-HCl buffer.
7. Repeat step 4 using PBS.
8. Wash polymer in PBS until the washings have negligible absorption at 280 nm.

Tube Absorption Procedure

1. Mix 2 volumes of serum with 1 volume of packed polymer and stir on a magnetic stirrer at 37° for 1 hr.
2. Centrifuge at 4500 rpm for 5 min.
3. Transfer supernatant to another tube and recentrifuge.
4. Remove the supernatant and test it for specificity.

NOTE: The polymer can be "regenerated" for further use by washing extensively with PBS followed by incubation with 3 *M* sodium thiocyanate, pH 6.6, for 30 min at room temperature to elute adsorbed protein. Wash the polymer finally with PBS and store at 4° in PBS containing 0.1% sodium azide.

Preparation of Immunoglobulin Fractions from Whole Serum

Precipitation with Rivanol and Ammonium Sulfate

Materials

Rivanol (2-ethoxy 6,9-diaminoacridine lactate)
Activated charcoal
Saturated ammonium sulfate solution
Isotonic saline

Procedure

1. Adjust antiserum to pH 8.5 by careful addition of 0.1 *N* NaOH.
2. For each 10 ml of antiserum add 35 ml of 0.4% Rivanol solution dropwise from a separating funnel. Stir the serum gently on a magnetic stirrer throughout.
3. Decant the supernatant (containing the immunoglobulins) into universal bottles and centrifuge in a bench centrifuge to remove remaining sediment.
4. Decant the supernatant into a conical flask and add activated charcoal (1–1.5 g per 100 ml) to decolorize the solution. Agitate gently for approximately 10 min.
5. Remove charcoal from the protein solution by filtering through a double layer of moistened filter paper (Whatman No. 42) in a Büchner funnel. Transfer filtrate to a beaker.
6. Add an equal volume of saturated ammonium sulfate solution dropwise from a separating funnel, stirring gently on a magnetic stirrer throughout.

7. When all the ammonium sulfate solution has been added, place the beaker at 4° for at least 6 hr to allow the immunoglobulin precipitate to flocculate.
8. Centrifuge at about 4000 *g* for 20 min, preferably in a refrigerated centrifuge, and discard the supernatant.
9. Dissolve the precipitate in a volume of saline approximately equivalent to half the volume of original antiserum.
10. Place the immunoglobulin solution in Visking tubing and dialyze extensively against several changes of saline to remove sulfate ions. (Alternatively, remove sulfate by chromatography on a Sephadex G-25 column.)
11. Check for residual sulfate ions by adding a few drops of the immunoglobulin solution to a tube containing a small volume of barium chloride solution. Any cloudiness indicates the presence of sulfate ions and the need for further dialysis.
12. Measure the volume of immunoglobulin solution and calculate the protein concentration by measuring the absorbance of a 1:25 dilution at a wavelength of 280 nm using a cuvette of 1 cm path length.

$$\text{concentration} = (OD_{280} \times 25)/1.34 \text{ mg/ml}$$

(The factor 1.34 can be used for the immunoglobulins of most animal species).

Precipitation with Caprylic Acid[24]

Materials

Acetate buffer, 60 m*M*, pH 4.0
Caprylic acid
Isotonic saline

Procedure

1. Add 2 volumes of acetate buffer to the antiserum in a beaker. Check and adjust the pH of the mixture to 4.8.
2. For each 10 ml of starting antiserum add 0.74 ml of caprylic acid dropwise. Stir the mixture continuously on a magnetic stirrer at room temperature.
3. Continue stirring for 30 min.
4. Centrifuge at 4000 *g* to remove the precipitate (or filter on a Büchner funnel).
5. Retain the supernatant (containing the immunoglobulin) and dialyze extensively against saline at 4°.

[24] N. Steinbuch and R. Audran, *Arch. Biochem. Biophys.* **134**, 279 (1969).

6. Measure the volume of the immunoglobulin solution and calculate the protein content as described above.

NOTE. Since the final volume of immunoglobulin solution is approximately three times the volume of starting antiserum, concentration is usually necessary. This may be achieved by ammonium sulfate precipitation as described above, pressure ultrafiltration, or dialysis against hypertonic polyethylene glycol. If the latter procedure is used, the immunoglobulin preparation should subsequently be redialyzed against saline to remove any polyethylene glycol that has diffused into the dialysis bag, thereby contributing to the absorbance at 280 nm.

Ion Exchange Chromatography

Immunoglobulins, in particular IgG, may be separated from whole serum by ion exchange chromatography. The technique relies upon differences in the net charge of serum proteins: at low ionic strength and neutral pH, IgG carries a neutral or slight net positive charge and will not be adsorbed to diethylaminoethyl (DEAE) cellulose, unlike all other serum proteins. Although the principle of the method remains the same for the serum proteins of different species, the exact conditions of pH and ionic strength required for good separation of IgG will vary. A method for preparation of rabbit IgG by ion exchange chromatography using a batchwise procedure is outlined below.

Materials

Diethylaminoethyl (DEAE) microgranular preswollen cellulose (Whatman DE-52)
Phosphate buffer, 5 m*M* pH 6.5

Procedure. The batchwise procedure of Stanworth[25] is used.

1. Equilibrate approximately 5 g of DEAE-cellulose with several changes of phosphate buffer.
2. Dialyze 20 ml of serum against phosphate buffer at 4° overnight.
3. Place the cellulose slurry in suitable containers such as universal bottles or large test tubes and centrifuge to sediment the particles. Check the pH of the supernatant buffer against that of the starting buffer to ensure that equilibration is complete. Discard the supernatant.
4. Add dialyzed antiserum to the packed cellulose and mix by gentle rotation for 1 hr at room temperature.
5. Centrifuge gently to sediment the cellulose, then carefully transfer the supernatant (containing immunoglobulin) to a clean container. Discard the cellulose.
6. Recentrifuge to remove any remaining cellulose, and decant the supernatant immunoglobulin solution.
7. Calculate the protein content as described previously.

If this preparation contains serum proteins other than immunoglobulin the process may be repeated using a fresh aliquot of equilibrated DEAE-cellulose.

[25] D. R. Stanworth, *Nature (London)* **188**, 156 (1960).

Preparation of Immunospecific (Affinity-Purified) Antibody

For some purposes it is necessary to use specific antibody rather than whole antiserum or a crude immunoglobulin fraction. Immunospecific antibody can be prepared by passing antiserum or a globulin fraction through an immunoadsorbent column containing antigen chemically coupled to an inert solid phase. Specific antibody combines with the immobilized antigen and can be eluted subsequently with "chaotropic" ions (such as thiocyanate) or low pH buffers. A method for preparation of human IgG immunoadsorbent and the elution of specific anti-IgG antibodies is given here.

Use of IgG-Sepharose Immunoadsorbent Prepared by Periodate Oxidation[26,27]

Materials

Sepharose CL4B
Sodium metaperiodate
Ethanediol
Isotonic saline
Carbonate–bicarbonate buffer, 0.1 *M*, pH 9.5
Phosphate-buffered saline 10 m*M*, pH 7.5 (PBS)
Sodium borohydride
Sephadex G-50, suspended in PBS
Sodium thiocyanate, 3 *M*, adjusted to pH 6.6

Procedure

ACTIVATION OF SEPHAROSE

1. Suck dry some of the Sepharose CL4B slurry. Weigh out 20 g of the gel and wash it with saline in a Büchner funnel containing two Whatman No. 54 filter papers.

[26] C. J. Sanderson and D. V. Wilson, *Immunology* **20**, 1061 (1971).
[27] T. J. G. Raybould and S. M. Chantler, *J. Immunol. Methods* **27**, 309 (1979).

2. Make up 40 ml of 10% sodium metaperiodate solution in distilled water.
3. Suck the Sepharose dry on the Büchner funnel and transfer the pad to the periodate solution. Mix or stir gently for 2–4 hr at room temperature.
4. Transfer the Sepharose slurry to a Büchner funnel containing two Whatman No. 54 papers. Wash quickly with saline to remove periodate.
5. Pour on 40 ml of 10% aqueous ethanediol, allowing the liquid to run through the gel very slowly to ensure thorough washing.
6. Wash the activated Sepharose finally with sodium carbonate–bicarbonate buffer and suck dry.

COUPLING OF ANTIGEN

1. Prepare 100 ml of IgG solution at a concentration of 1.0 mg/ml in sodium carbonate–bicarbonate buffer.
2. Add the activated Sepharose to the IgG solution and mix or stir gently for 18 hrs at room temperature (or 4° if preferred).
3. Transfer the slurry to a Büchner funnel containing two Whatman No. 54 papers. Suck dry and wash with PBS.
4. Prepare 20 ml of a 5 mg/ml aqueous sodium borohydride solution.
5. Transfer the Sepharose pad to the borohydride solution and mix or stir gently for 2 hrs at room temperature. (CARE: Borohydride reduction is accompanied by evolution of hydrogen and should be carried out in a loosely stoppered vessel in a well ventilated area).
6. Transfer the gel to a Büchner funnel containing two Whatman No. 54 papers and suck dry. Wash extensively with PBS, and finally resuspend in PBS to desired concentration. The gel is now ready for use.

PREPARATION OF IMMUNOADSORBENT COLUMN

1. Clamp a column (approximately 1.5 cm × 40 cm) to a stand, and with the outlet closed run a small volume of PBS into the column.
2. Pour the Sephadex G-50 suspension into the column and allow to settle until approximately 1 cm of column length is filled. Open the outlet to allow a flow of PBS, which facilitates column packing. Add more Sephadex slurry to give a packed volume of one-third of the column length, with a reasonable depth of PBS above the packed Sephadex. Close the outlet.
3. Pour the IgG–Sepharose slurry into the column carefully so as not to disturb the surface of the Sephadex and allow it to settle in a separate layer on top of the Sephadex.
4. Cut a circle of Whatman No. 54 filter paper the same size as the internal diameter of the column and allow to float onto the settled surface of the IgG–Sepharose. This prevents disturbance of the surface of the column during subsequent sample and buffer applications.
5. Open the column outlet to allow excess buffer to run through and wash the column contents by passage of PBS until the absorbance of the effluent at 280 nm is equivalent to that of washing buffer.

APPLICATION OF ANTISERUM OR GLOBULIN SAMPLE

1. Dialyze 1 ml of the serum or globulin solution against PBS overnight at 4°.
2. Open the column outlet to allow the head of buffer to pass into the gel. Close the outlet.
3. Apply the dialyzed sample to the top of the column, taking care to avoid disturbance of the Sepharose.
4. Open the outlet and allow the sample to run into the Sepharose column, closing the outlet when all the liquid has been absorbed.
5. Run PBS onto the top of the column and allow to flow through slowly by opening the outlet slightly. Ensure that a head of PBS is always present to avoid drying out.
6. Unadsorbed serum proteins will pass through the column and can be detected by a suitable monitor. When all the protein has emerged allow the remaining head of PBS to pass into the column and then close the outlet.

ELUTION OF BOUND ANTIBODY

1. Gently apply 5 ml of sodium thiocyanate solution to the column and allow to run into the gel by opening the outlet.
2. As soon as the thiocyanate solution has entered the gel, close the outlet, apply PBS, reopen the outlet and allow PBS to flow continuously through the column as previously. Eluted antibody contained in the thiocyanate solution will pass through the Sepharose and into the lower, Sephadex portion of the column. The molecular sieving properties of the Sephadex will serve to separate antibody rapidly from the thiocyanate and reduce the risk of denaturation.
3. Collect fractions containing the antibody, pool, and concentrate to approximately 5 mg/ml.
4. Measure the volume and protein content. Store frozen or freeze-dried in suitable size aliquots.

NOTE. This method of purification will select all antibodies reacting with the antigen on the immunoadsorbent, including any that may cross-react with other antigens by virtue of shared determinants. If such antibodies are likely to be present (as, for instance, will be the case in antisera raised against whole IgG), they should be removed by straightforward ab-

sorption as described in a previous section: Absorption of Nonspecific Antisera. Absorption may be carried out before or after preparation of the immunospecific antibody, but the former is to be preferred for logistic reasons.

Preparation of Fluorochrome and Enzyme-Labeled Antibodies

Selection of Antisera for Conjugation

Satisfactory conjugates can be prepared only from potent antisera of the required immunological specificity; it is important therefore that the purest available antigen be employed as immunogen and that multiple injections be given to ensure the production of antisera in which the ratio of antibody globulin to nonantibody globulin is high.

Ideally, antiserum should be selected on the basis of tests of potency and specificity carried out prior to labeling. This preliminary evaluation may conveniently be performed by titration in conventional gel diffusion and assessment of specificity in immunoelectrophoresis. If the lack of a suitable soluble antigen makes such tests impossible, indirect immunofluorescent or immunoenzyme tests should be done utilizing a range of dilutions of pre- and postimmunization sera as the intermediary layer followed by the appropriate labeled anti-species immunoglobulin. Test samples, which may be histological preparations or cell films should be appropriately prepared (some prior knowledge of the system is almost essential) and should represent both "positive" (antigen containing) and "negative" (non-antigen containing) materials. Antisera exhibiting the highest level of activity and specificity should be selected for labeling.

Labeling should be carried out on immunoglobulin preparations derived from the selected antisera, so as to maximize the proportion of specific antibody to total protein and hence reduce non-specific activity in the final reagent. Immunoglobulin can be prepared by any of the methods described above, but only in the most demanding systems will it be necessary to prepare immunospecific antibody rather than a crude immunoglobulin fraction.

Fluorescein Labeling of Antibody Globulins

Materials

Fluorescein isothiocyanate, isomer I (FITC)
Carbonate–bicarbonate buffer, 0.1 *M*, pH 9.0
Immunoglobulin preparation (10 mg/ml in saline)
Phosphate-buffered saline, pH 7.5 (PBS)
Sephadex G-50 medium

Procedure

1. Prepare a solution of FITC in carbonate–bicarbonate buffer to give a solution containing 1 mg of dye per milliliter.
2. Place a measured volume of the immunoglobulin solution in a small beaker and cool to 4°. Place on magnetic stirrer.
3. Add one-tenth volume of carbonate–bicarbonate buffer.
4. Add one-tenth volume of FITC solution dropwise while stirring the immunoglobulin solution at 4° (approximately 1 mg of dye per 100 mg of protein).
5. Check pH after addition of FITC and if necessary adjust to pH 9.0 with 0.1 *N* NaOH.
6. Cover reaction vessel and stir *gently* at 4° overnight. (Alternatively the reaction can be carried out at room temperature for 1–2 hr if the volume to be labeled is less than 20 ml.)

Removal of unreacted free FITC is preferably performed by dialysis followed by gel filtration chromatography on Sephadex G-50 (medium).

7. Dialyze conjugate against several changes of phosphate-buffered saline (PBS).
8. Prepare Sephadex G-50 column equilibrated with PBS such that the packed volume is at least six times the volume of conjugate to be applied. Allow a disc of filter paper, cut to fit the dimensions of the column, to float onto the top of the column. This facilitates the even application of conjugate.
9. Allow the PBS to run through the column until no buffer remains above the top of the column.
10. Stop the flow of buffer and apply the conjugate.
11. Allow the conjugate to flow into the column by opening the tap. When all the conjugate has passed into the column elute with PBS.
12. Collect the first colored peak to emerge (this contains the labeled immunoglobulins) and concentrate to the original conjugate volume.
13. Conjugates can be stored at 4° or in aliquots at −20° after the addition of a preservative such as 0.1% sodium azide. Repeated freezing and thawing is to be avoided.

Peroxidase Labeling of Antibody Globulins

Although a variety of methods can be used for coupling enzymes to antibody,[28] the conjugation procedures most commonly used with horse-

[28] S. Avrameas, T. Ternynck, and J. L. Guesdon, *Scand. J. Immunol.* **8**, *Suppl.* 7, 7 (1978).

radish peroxidase (HRP) are the two-stage glutaraldehyde[29] and periodate oxidation[30] methods. In the former procedure peroxidase is first mixed with an excess of the dialdehyde glutaraldehyde, which reacts with free amino groups of the enzyme via only one of its active aldehyde groups. After gel filtration chromatography to remove excess glutaraldehyde, the activated enzyme is mixed with the immunoglobulin preparation to allow the free aldehyde group to combine with an amino group of the immunoglobulin. Conjugates prepared in this way have been shown to contain a homogeneous derivative[29,31] with a molecular weight of 90,000, but the coupling efficiency is poor at around 25% and 5% for antibody and enzyme, respectively.[32] The low efficiency in this system appears to be due to the relative paucity of reactive amino groups in HRP. In contrast the periodate oxidation method of conjugation[30,33] is not dependent on the presence of reactive amino groups but relies upon the generation of active aldehyde groups after periodate oxidation of the carbohydrate moiety of peroxidase. These aldehyde groups combine with the amino groups of added immunoglobulin to form Schiff bases, which are subsequently stabilized by reduction with sodium borohydride. Conjugates prepared by this procedure contain high molecular weight derivatives,[30,32] but the coupling efficiency is increased to approximately 60% for both antibody and enzyme.[34]

Recent studies using a modification of the method described by Kato *et al.*[35] have shown that peroxidase can be satisfactorily coupled to antibody by coupling via sulfhydryl groups introduced into both the immunoglobulin and enzyme structures.[36] Conjugates prepared in this way contain active derivatives that are heterogeneous in relation to molecular weight but retain good enzyme and antibody activity.[37]

Glutaraldehyde Conjugation Method[28]

Materials

Horseradish peroxidase RZ 3.0
Stock solution of glutaraldehyde, 25% in water
Phosphate buffer, 0.1 *M*, pH 6.8
Sephadex G-25
Isotonic saline
Immunoglobulin preparation, 5 mg/ml in saline
Carbonate–bicarbonate buffer, 0.5 *M*, pH 9.5
Lysine solution, 1.0 *M* pH 7
Phosphate-buffered saline, pH 7.5 (PBS)
Saturated ammonium sulfate
Glycerol

Procedure

1. Dissolve 10 mg of peroxidase in 0.2 ml of a freshly prepared 1:25 dilution of the stock glutaraldehyde solution in phosphate buffer and allow to stand at room temperature for 18 hr.
2. Pass through Sephadex G-25 column equilibrated with saline to remove excess glutaraldehyde.
3. Collect the brown fractions, which contain the activated peroxidase, pool, and concentrate to 1 ml.
4. Add 1 ml of immunoglobulin solution (previously dialyzed against saline) to the peroxidase solution.
5. Add 0.2 ml of carbonate–bicarbonate buffer and leave for 24 hr at 4°.
6. Add 0.1 ml of lysine solution and leave the mixture at 4° for 2 hr.
7. Dialyze against several changes of PBS at 4°. If desired remove free enzyme by precipitation with saturated ammonium sulfate as described in steps 8–10.
8. Add an equal volume of saturated ammonium sulfate to the conjugate and allow to stand at 4° for 30 min.
9. Centrifuge for 20 min at 4000 *g* and discard supernatant.
10. Dissolve precipitate in approximately 1 ml of saline and dialyze extensively against several changes of PBS. (Alternatively, sulfate ions may be removed by gel filtration chromatography on Sephadex G-50.)
11. Preserve by adding an equal volume of glycerol, and store at 4°.

Periodate Oxidation Conjugation Method

Two procedures have been described by Nakane and co-workers. In the first of these[30] free amino groups on the peroxidase are blocked by fluorodinitrobenzene (FDNB) treatment prior to the production of active aldehyde groups by periodate oxidation. A recent modification of this method, described here, omits FDNB blocking and recommends periodate oxidation of the enzyme at low pH prior to coupling with immunoglobulin.[33]

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[36] P. D. Weston, J. A. Devries, and R. Wrigglesworth, *Biochim. Biophys. Acta* **612**, 40 (1980).
[37] S. M. Chantler and L. S. Cooper, unpublished observations, 1978.

Materials

Horseradish peroxidase RZ 3.0 (HRP)
Sodium metaperiodate (freshly prepared), 0.1 *M*
Acetate buffer, 1 m*M*, pH 4.4
Carbonate–bicarbonate buffer, 10 m*M*, pH 9.5
Immunoglobulin preparation
Carbonate–bicarbonate buffer, 0.2 *M*, pH 9.5
Sodium borohydride
Sephacryl S-200
Phosphate-buffered saline pH 7.5 (PBS)

Procedure

1. Dissolve 4 mg of HRP in 1 ml of distilled water.
2. Add 0.2 ml of freshly prepared periodate to the enzyme solution and stir for 20 min at room temperature.
3. Dialyze against acetate buffer overnight at 4°.
4. Prepare globulin solution containing 8 mg of protein in 1 ml of 10 m*M* carbonate–bicarbonate buffer.
5. Adjust activated HRP solution to approximately pH 9 by addition of 20 μl of 0.2 *M* carbonate–bicarbonate buffer.
6. Immediately add the globulin preparation to the HRP–aldehyde and stir for 2 hr at room temperature.
7. Add 0.1 ml of freshly prepared sodium borohydride solution containing 4 mg/ml and leave at 4° for 2 hr.
8. Separate unreacted enzyme from the mixture by chromatography on a column of Sephacryl S-200 equilibrated with PBS or by salt precipitation with ammonium sulfate as described above.
9. If purification of conjugates is performed by gel chromatography, the appropriate fractions should be pooled and concentrated prior to storage at −20°. Addition of albumin (10 mg per milliliter of conjugate) or an equal volume of glycerol prior to freezing in small aliquots is recommended. Repeated freezing and thawing should be avoided.

Evaluation of Conjugates

A variety of tests should be used to determine the efficiency of conjugation and the suitability of the conjugate in use. The extent of the testing performed, particularly with respect to specificity, will vary with the intended use of the reagent.

Efficacy of labeling can be determined very simply by measuring the absorbance of the conjugate both at the 280 nm protein peak and at the maximum absorbance wavelength of the label used. For immunohistological studies the ratio of OD_{495} to OD_{280} for fluorescein-labeled reagents should lie between 0.6 and 0.9; and the ratio of OD_{403} to OD_{280} for peroxidase-labeled conjugates, between 0.3 and 0.6. This test, however, fails to show whether biological activity is present in the conjugate. This should be determined initially by using the conjugate as the antibody in appropriate gel diffusion or immunoelectrophoresis tests (if a suitable soluble antigen preparation is available), followed by testing in the immunofluorescent or immunoenzyme system in which it is to be used. Performance testing by titration (direct method) or chessboard titration (indirect method) is *essential* in order to select the optimal working dilution of the reagent and to assess its specificity under working conditions. Tests of immunological specificity carried out by other methods (e.g., gel diffusion) are irrelevant and may even give misleading results because of the widely varying sensitivity shown by different test systems.[38]

Antibody Production by Lymphocyte Hybridomas[38a]

Conventional immunization by injection of antigen into an animal stimulates the production of a heterogeneous population of antibodies that differ in respect of both their affinity and their specificity. Although the immunization procedure or prior treatment of the recipient may be manipulated to favor the production of antibodies of predominantly high or low affinity, the specificity of the antibody response is less amenable to control and antibodies directed against each of several antigenic determinants present in the immunogen will usually be present. The extent of this heterogeneity of response will differ not only among members of different species but also in individual animals of the same species despite the use of identical immunogen preparations and immunization schedules. These biological factors influence both the ease and reproducibility with which antisera of the desired immunological specificity can be prepared. The application of cell fusion techniques for *in vitro* production of antibodies of defined-specificity offers a significant potential alternative to conventional methods of reagent antibody production.

In 1973, Cotton *et al.*[39] successfully fused cells of two plasmacytoma lines to produce hybrid cells capable of synthesizing both myeloma proteins. Subsequently, hybrid cells derived by fusion of a murine myeloma with spleen cells from appropriately immunized donors were shown to se-

[38] S. M. Chantler and M. Haire, *Immunology* **23**, 7 (1972).

[38a] The authors wish to acknowledge the helpful criticism of Dr. Jane Hewitt during the preparation of this section.

[39] R. G. H. Cotton, D. S. Secherand, and C. Milstein, *Eur. J. Immunol.* **3**, 135 (1973).

crete antibodies against the immunogen used.[40] These hybrid cells (hybridomas) could be grown in tissue culture, producing antibodies of defined specificity *in vitro*; alternatively, antibody secretion could be obtained *in vivo* by inoculation of the hybridoma cells subcutaneously or intraperitoneally into syngeneic recipients. This approach thus offered the possibility of the production of monoclonal antibody of defined specificity by selective cloning procedures, avoiding the need for highly purified immunogen or elaborate antibody purification procedures.

Although it is now well established that the fusion of mouse myeloma cells and antibody-secreting splenic lymphocytes is an effective means of producing homogeneous antibody of defined specificity, a number of technical variables remain. The same basic principles are applicable to many systems, but fairly extensive preliminary investigation is required to define the optimal conditions, particularly in relation to the choice of immunization schedule and donor species used. Investigators interested in detailed methodology should refer to the recent proceedings of a workshop on lymphocyte hybridomas.[41]

Choice of Fusion Partners

The myeloma line selected should exhibit good growth characteristics *in vitro*, a high fusion frequency (one hybrid per 10^5 to 10^6 normal cells) and should be sensitive to the selective medium HAT. If the cell line is lacking in either of the enzymes hypoxanthine guanine phosphoribosyltransferase (HGPRT) or thymidine kinase (TK), growth in this selective medium (which contains hypoxanthine, aminopterin, and thymidine) will be impossible.[42] Only after hybridization with a normal cell containing the enzymes can DNA synthesis and growth occur; thus hybrid cells alone survive in the selective medium. A limited number of myelomas exhibiting these features are available, and the one most commonly used is P3-X63Ag8 of BALB/c origin.

Hybrids obtained by fusion of an antibody-secreting normal cell and a myeloma cell such as the above produce specific antibody together with the myeloma protein and the products of mixed genetic combinations. This heterogeneous immunoglobulin production may not always pose a problem, but in applications where greater purity is necessary the difficulty can be avoided by using a nonsecreting myeloma line that produces

[40] G. Köhler and C. Milstein, *Nature (London)* **256**, 495 (1975).
[41] F. Melchers, M. Potter, and N. L. Warner, eds., "Lymphokine Hybridomas," *in Curr. Top. Microbiol. Immunol.* **81**. (1978).
[42] J. W. Littlefield, *Science* **145**, 709 (1964).

no immunoglobulin of its own but still supports the synthesis of spleen cell-derived immunoglobulins.[43,44]

The phylogenetic relationship between the cells utilized in hybridization studies determines the functional success of the hybrids produced. Murine myeloma lines have been successfully fused to both syngeneic and allogeneic mouse spleen cells[45,46] and to rat spleen cells,[47] but fusion with human lymphocytes and with cells of rabbit or frog origin has been less successful.[48] Recently Galfre and his colleagues[49] have described a rat myeloma line that has been successfully fused to rat spleen cells, but as yet no suitable human myeloma lines are available. The ontological derivation of potential fusion partners is also important.[50] It appears that optimal results are dependent upon fusion with cells of the B lymphocyte series at an appropriate stage of differentiation. Although the exact characteristics of the cell have not been identified, an activated B lymphocyte at an early stage of differentiation appears to be preferable. It follows therefore that selection of fusion partners of compatible phylogeny and ontogeny together with preselection of suitably differentiated B lymphocytes will increase the success rate of obtaining functional hybridomas. In practice, splenic cells from immunized mice have been most extensively used in experimental work because of the availability of suitable murine myelomas.

Although the myeloma line (P3-X63Ag8) commonly used in fusion studies is derived from the BALB/c mouse strain, it is not essential to use this inbred strain as a source of donor cells. Instead, it is preferable to use a strain that provides the best response to the immunogen in question; however, if it is intended finally to inoculate the hybrid clones into animals in order to produce antibodies *in vivo*, then clearly the recipient animal must be histocompatible. This can be achieved by using, as recipients, F_1 hybrids of BALB/c and the strain selected for initial immunization.

[43] M. Schulman, C. D. Wilde, and G. Köhler, *Nature (London)* **276**, 269 (1978).
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[48] G. Köhler and M. J. Schulman, *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter and N. L. Warner, eds.), *Curr. Top. Microbiol. Immunol.* **81**, 143 (1978).
[49] G. Galfre, C. Milstein, and B. Wright, *Nature (London)* **277**, 131 (1979).
[50] P. Coffino, B. Knowles, S. Nathenson, and M. D. Scharff, *Nature (London), New Biol.* **231**, 87 (1971).

Immunization Procedure

In most somatic cell hybridization studies the potential spleen cell donor is immunized in order to increase the proportion of cells producing specific antibody. This enrichment of functionally active cells has been shown to increase the percentage of hybridomas exhibiting the desired specific antibody activity. The type of immunization schedule adopted will depend upon the physical nature of the antigen and its immunogenicity, so that the variables, such as use of adjuvant, route of injection, and the timing of injections, will differ in different studies. Immunization commonly involves an initial subcutaneous injection of immunogen followed by a booster intravenous injection. The animals are tested 2–5 days after the boost, and a good responder is given a second intravenous injection, spleen cells being harvested 2–5 days later.

Preparation of Spleen Cells

Separation of nucleated cells from red blood cells present in the spleen cell suspension is rarely performed. Spleen cells are washed twice in serum-free medium, the yield from one spleen being approximately 1×10^8 nucleated cells. A ratio of 10 spleen cells : 1 myeloma cell is used for fusion. If it is possible to enrich the proportion of plaque-forming cells in the spleen suspension—for instance, by rosetting with antigen-labeled red blood cells followed by centrifugation in Ficoll-Isopaque—the ratio used for fusion may be reduced to 1 : 1. Such an enrichment procedure not only decreases the number of cells that need to be distributed into individual culture wells after fusion, but also increases the percentage of hybridomas that secrete antibody of the desired specificity, thereby reducing the number of tests performed in the selection of appropriate hybrid clones at a later stage.

Cell Fusion

In early studies fusion was promoted by the use of Sendai virus, but more recently polyethylene glycol (PEG) of molecular weight 1000–6000 has been preferred. The sediment of spleen and myeloma cells is gently resuspended in the small volume of washing medium remaining after centrifugation, and approximately 2 ml of 50% PEG solution diluted in the serum-free medium is added. After incubation at 37° for 1 min, the cell mixture is diluted *slowly* with medium, approximately 5 ml being added over a period of 5 min. The suspension is then centrifuged and resuspended in the selective HAT medium (containing serum) to a final density of approximately 10^6 cells per milliliter. This procedure yields approximately 100 ml of suspension from one spleen.

Growth of Hybrid Cells

The fused cells, suspended in HAT medium, are seeded into individual tissue culture wells, putting approximately 10^6 cells into each well. Unfused myeloma cells cannot grow in this selective medium, and normal spleen cells are incapable of prolonged growth, so only the hybrid cells survive. These "microcultures" are examined periodically, and those showing growth visible over approximately 30% of the base of the individual wells are tested for specific antibody activity, this stage being reached in successful wells between 7 and 20 days after seeding. The percentage of wells showing growth will depend on the number of cells originally introduced: approximately 90% of wells exhibit growth when 10^6 cells are placed in each culture well. The majority of the wells will contain multiple clones derived from different parent hybrid cells, the products of many which are irrelevant to the particular study. The proportion of wells containing functional hybrids of the desired specificity will vary considerably, but approximately 5% of those showing growth may contain appropriate hybrids.

Evaluation of Activity of Hybrid Products

The supernatants obtained from individual culture wells exhibiting growth must be tested to determine whether any hybrids present in that culture are secreting antibody of the required specificity. Since the level of immunoglobulin secretion is low (approximately 10–50 μg/ml) and the number of wells to be tested may be relatively large, it is essential that highly sensitive and specific assays that are readily performed on small volumes of supernatants be used for screening. Radioimmunoassays are most widely used, but hemagglutination, hemagglutination inhibition, and (in cases where localization of activity is relevant) immunofluorescence and immunoenzyme procedures have been applied.

Cloning of Active Hybrids

As previously mentioned, culture wells containing antibody of the appropriate specificity may contain a heterogeneous population of hybrid cells secreting a variety of products. Individual hybrid cells can be separated only by additional cloning procedures, either by growth in soft agar or by using the limiting dilution method.

Cloning by the soft agar method is carried out in petri dishes 3 cm in diameter that contain a layer of normal spleen "feeder" cells (10^6 per plate) in 5% agar, over which is then layered a dilution of the hybrid cells (obtained from positive wells) suspended in a medium containing 20% fetal calf serum in 2.5% agar. A range of different dilutions of the hybrid

cells may be treated in this way. After incubation, individual clones of cells are detectable within 1–2 weeks. These discrete colonies are then transferred to microculture wells and their products are again tested for activity of the required specificity. Cultures exhibiting appropriate activity are immediately recloned at least twice in order to select stable functional cell lines, which are stored by freezing in vials or transferred to larger culture vessels.

The limiting dilution method of cloning involves culturing serially diluted suspensions of hybrid cells together with normal spleen cells, each dilution being set up in 6–12 wells. The average number of hybrid cells dispensed in each series lies between 240 and 0.1 cells per well. At high cell levels growth is observed in most wells, but statistical considerations suggest that if only one-third of the wells seeded at a particular cell dilution show growth, then it is highly probable that the cells growing within each of the individual wells are derived from a single parent cell. These wells are then tested for antibody activity, and the cellular contents are recloned to establish functional stability in the same way as those derived by soft agar cloning procedures.

Antibody Production

Once stable hybrid clones secreting antibody of defined specificity have been isolated, methods of obtaining maximal amounts of antibody become important. These may involve *in vitro* culture or *in vivo* growth in a suitable recipient. The hybridomas may be maintained in continuous culture *in vitro* for several months at a cell density within the range of 10^4 to 4×10^6 cells per milliliter. Under these conditions an antibody yield of 10–100 μg/ml can be obtained, but in most cases loss of functional activity eventually occurs. The reason for such functional instability is not clear, but it is likely to be due to loss of chromosomes during a period of time in culture. For this reason *in vitro* antibody production is more satisfactorily performed in limited rather than continuous culture, selected stable clones being stored by freezing at an early stage in their life cycle so that a new vial of cells can be thawed when required to initiate a fresh culture.

As an alternative, antibody can be produced *in vivo*. Many cultured hybridomas have been successfully transplanted to genetically compatible recipients,[45,49,51] and hybridomas derived from cells of nonmurine origin have been successfully transplanted to athymic nude mice.[52] *In vivo* antibody production is achieved by inoculating the cloned, hybrid cells

[51] T. Pearson, G. Galfre, A. Ziegler, and C. Milstein, *Eur. J. Immunol.* **7**, 684 (1977).
[52] H. Koprowski, Z. Steplewski, D. Herlyn, and M. Herlyn, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3405 (1978).

subcutaneously or into the peritoneal cavity. If the latter route is used, mineral oil is given several days prior to inoculation in order to encourage the production of ascitic fluid. The level of antibody obtained by *in vivo* culture is reported to be 100- to 1000-fold greater than for *in vitro* culture.[39,45,49,51]

In assessing the efficacy of *in vitro* versus *in vivo* production of reagent antibody, consideration must be given both to the relative concentrations of antibody and to the volumes obtainable. Subcutaneous inoculation of cells in a mouse yields approximately 1 ml of serum 2 weeks later; the yield of ascitic fluid harvested 7–14 days after intraperitoneal injection is between 5 and 15 ml. As the concentration of antibody produced in this way is at least 100-fold greater than in tissue culture, 10 ml of ascitic fluid from one mouse would be equivalent to at least 1 liter of tissue culture fluid. In this context, the recent description of hybridomas produced by fusion of a rat myeloma line with rat spleen cells is likely to be of considerable practical significance because of the larger volume of serum obtainable following inoculation of hybridomas in these rodents.[49]

Nonhybridoma Techniques

The production of a thriving, functional hybridoma is dependent on a close phylogenetic relationship between the two parent cell lines; the only species to have provided suitable cell lines so far are mice and rats. An alternative approach to the production of nonrodent antibodies in cell culture is provided by the transformation of B lymphocytes on exposure to Epstein–Barr virus (EBV). Adult human peripheral blood cells exposed to EBV have been shown to release polyclonal secretory immunoglobulin.[53] Cultures of human peripheral blood lymphocytes exposed to antigen (sheep red blood cells) and EBV produce specific antibody.[54] Preselection of human peripheral blood lymphocytes exhibiting surface binding of tetanus toxoid and the hapten NNP (4-hydroxy-3,5-dinitrophenacetic acid) followed by viral transformation has been shown to yield cells capable of antibody production *in vitro*.[55,56] Although the cultures have been shown to be active for some months, methods of increasing both the yield of antibody (at present only some 10 ng per milliliter of culture fluid) and long-term stability have yet to be devised.[57] Attempts to establish stable spe-

[53] A. Rosen, P. Gergely, M. Jondal, and G. Klein, *Nature (London)* **267**, 52 (1977).
[54] A. L. Luzzatti, H. Hengartner, and M. H. Schreier, *Nature (London)* **269**, 419 (1977).
[55] V. R. Zurawski, E. Haber, and P. H. Black, *Science* **199**, 1439 (1978).
[56] M. Steinitz, G. Klein, S. Koskimies, and O. Makel, *Nature (London)* **269**, 420 (1977).
[57] V. R. Zurawski, S. E. Spedden, P. H. Black, and E. Haber, *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter, and N. L. Warner, eds.), *Curr. Top. Microbiol. Immunol.* **81**, 152 (1978).

cific antibody-secreting cell lines by somatic hydridization with the murine myeloma P3-X63Ag8 have been unsuccessful.[58]

Summary

The successful fusion of normal and neoplastic lymphocytes has laid the foundation for the production of a variety of antibody specificities of practical relevance in research and diagnosis. The technical problems associated with this approach should not be underestimated, but one cannot fail to recognize the enormous range of applications that lie ahead once these problems have been overcome.

[58] H. Hengartner, A. L. Luzzatti, and M. Schreir, *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter, and N. L. Warner, eds.), *Curr. Top. Microbiol. Immunol.* **81**, 92 (1978).

[6] Preparation of Fab Fragments from IgGs of Different Animal Species

By MICHAEL G. MAGE

The light and heavy polypeptide chains of the IgG molecule are folded into a series of globular regions called domains[1] (Fig. 1). The portion of the polypeptide chain between the Cγ1 and Cγ2 domains of the heavy chain, known as the "hinge region,"[2] is relatively accessible to proteolytic enzymes. When whole IgG molecules are incubated with the proteolytic enzyme papain, in the presence of low concentrations of sulfhydryl compounds, one or more peptide bonds in the hinge region are split,[3] leading to the release of the Fab and Fc fragments (Fig. 1).

The Fab fragments of IgG antibodies thus consist of the light chain and the V_H and Cγ1 domains[1] of the heavy chain. Fab fragments are univalent, in that each fragment contains a single antibody combining site, composed of parts of the variable regions (V_L and V_H) of the light and heavy chains. Because of their univalency, Fab fragments can be used to advantage in procedures where it is desirable to bind antigen to antibody in solution without cross-linking or precipitation or to bind to antigen on cell surfaces without producing "patching" or "capping."[4]

[1] G. M. Edelman and W. E. Gall, *Annu. Rev. Biochem.* **38**, 415 (1969).
[2] D. S. Smyth and S. Utsumi, *Nature (London)* **216**, 332 (1967).
[3] S. Zappacosta, A. Nisonoff, and W. J. Mandy, *J. Immunol.* **100**, 1268 (1968).
[4] F. Loor, L. Forni, and B. Pernis, *Eur. J. Immunol.* **2**, 203 (1972).

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[5] H. B. Dickler,
[6] V. P. Butler, J
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[7] R. R. Porter,
[8] M. Potter, M
[9] B. Benacerra
[10] E. T. Harrise